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Evaluation of different feeding protocols for larvae of Atlantic bluefin tuna (*Thunnus thynnus* L.)

Mónica B. Betancor[a,*], Aurelio Ortega[b], Fernando de la Gándara[b], Jose L. Varela[c], Douglas R. Tocher[a], Gabriel Mourente[c]

[a] Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, FK9 4LA Stirling, Scotland, UK
[b] Planta Experimental de Cultivos Marinos, Instituto Español de Oceanografía (IEO), 30860 Puerto de Mazarrón, Murcia, Spain
[c] Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, 11510 Puerto Real, Cádiz, Spain

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ABSTRACT

Mass mortality is still one of the main constraints in larval rearing of Atlantic bluefin tuna (*Thunnus thynnus* L.; ABT). Early data related to the feeding sequence of ABT larvae suggested that mortality observed during the first stages of life could be partly due to nutritional deficiencies. Previous studies demonstrated that copepods appeared to be a superior live prey compared to rotifers during the first 2 weeks of life. Our overarching aim was to evaluate different feeding strategies during first feeding of ABT larvae from a performance, compositional and molecular perspective. In order to do so, two groups of ABT larvae were fed with either copepod (*Acartia tonsa*; C) nauplii or rotifers (*Brachionus rotundiformis*; R) enriched with Algamac 3050® from mouth opening to 13 days after hatching (dah). After this, the group C-larvae was fed either *Artemia* enriched with Algamac 3050® (CA), *Acartia* nauplii and copepodites (CC) or sea bream (*Sparus aurata*) yolk-sac larvae (CY), while the R group were fed on *Artemia* enriched with Algamac 3050® (RA) up to 18 dah. At 13 dah, larvae fed copepods (C) had grown better than those fed enriched rotifers (R) although there were no significant differences in survival. ABT larvae fed R accumulated highest eicosapentaenoate (EPA) but lowest docosahexaenoate (DHA) and total n-3 long-chain polyunsaturated fatty acids (LC-PUFA) than C-fed larvae, reflecting the dietary contents. There was no activation in the expression of the enzymes involved in EPA and DHA biosynthesis. However, the different live prey showed regulation of transcription factor, digestive enzyme, lipid metabolism and oxidative stress genes. At 18 dah, larvae fed CY and CA treatments were largest in size, with larvae fed RA displaying the lowest growth, with no significant differences in survival among the dietary treatments. The highest DHA contents were found in ABT larvae fed CC and CY, whereas the lowest contents were found in RA-fed larvae. Indeed, larvae fed RA showed the highest level of the intermediate product n-3 docosapentaenoate, which could reflect increased activity of the biosynthetic pathway although this was not supported by gene expression data.

1. Introduction

Regardless of the efforts and progress that have been made in the Mediterranean area in the larval rearing of Atlantic bluefin tuna (ABT; *Thunnus thynnus*), a number of challenges and issues continue to restrict the hatchery production of ABT fingerlings in commercial quantities with early "mass mortality" during first feeding being a common occurence (De la Gándara et al., 2016; Van Beijnen, 2017). Aside from

Abbreviations: ABT, Atlantic bluefin tuna; *aco*, acyl coA oxidase; *alp*, alkaline phosphatase; *amy*, amylase; *anpep*, amino peptidase; ARA, arachidonic acid (20,4n-6); *bal1*, bile salt activated lipase 1; *bal2*, bile salt activated lipase 2; *cat*, catalase; *cpt1*, carnitine palmitoyl transferase I; dah, days after hatch; DHA, docosahexaenoic acid (22:6n-3); DPA, docosapentaenoic acid (n-3 or n-6); EPA, eicosapentaenoic acid (20:5n-3); *elovl5*, fatty acyl elongase 5; *fabp2*, fatty acid binding protein 2 (intestinal); *fabp*4, fatty acid binding protein 4 (adipocyte); *fabp7*, fatty acid binding protein 7 (brain-type); *fads2d6*, delta-6 fatty acyl desaturase; *fas*, fatty acid synthase; *gpx1*, glutathione peroxidase 1; *gpx4*, glutathione peroxidase 4; *hmgcl*, 3-hydroxy-3-methylglutaryl-CoA lyase; LA, linoleic acid (18:2n-6); LNA, α-linolenic acid (18:3n-3); LC-PUFA, long-chain polyunsaturated fatty acid; LPC, lyso phosphatidylcholine; *lpl*, lipoprotein lipase; *lxr*, liver X receptor; *myhc*, myosin heavy chain; PBT, Pacific bluefin tuna; *pl*, pancreatic lipase; *pla2*, phospholipase A_2; *ppara*, peroxisome proliferator-activated receptor alpha; *pparγ*, peroxisome proliferator-activated receptor gamma; *rxr*, retinoid X receptor; *sod*, superoxide dismutase; *srebp1*, sterol regulatory element-binding protein 1; *srebp2*, sterol regulatory element-binding protein 2; TF, transcription factor; *tropo*, tropomyosin; *tryp*, trypsin.

* Corresponding author.
E-mail address: m.b.betancor@stir.ac.uk (M.B. Betancor).

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improvement of zootechnical aspects of the culture system focussed on broodstock management and egg and larval production and quality, the refinement of the live food trophic chain (prey size, sequence and nutritional quality) is necessary until a reliable artificial commercial diet is fully developed. Currently, ABT larval first feeding is attained using either enriched rotifers (preferably *Brachionus rotundiformis*) and/or copepod nauplii (*Acartia tonsa*) as initial live prey (De La Gándara et al., 2010, 2016; Betancor et al., 2017a,b). Previous studies have shown that copepods were better live prey for first feeding ABT based on growth and survival data (Betancor et al., 2017a). In addition, broodstock nutrition was also identified as a possible factor explaining differences in growth performance and lipid metabolism observed between larvae from different year classes (Betancor et al., 2017a).

The metabolism and deposition of lipids is a complex process in fish, involving multiple pathways such as lipogenesis, β–oxidation and biosynthesis of long–chain polyunaturated fatty acids (LC-PUFA) (Ayisi et al., 2018). In a recent trial where ABT larvae were fed on rotifers enriched with five commercial products or copepods nauplii, responses in lipid gene expression, which are a consequence of differences in dietary lipid contents and fatty acid compositions, appeared to indicate that lipid levels provided by enriched rotifers exceeded ABT requirements (Betancor et al., 2017b). This study corroborated that copepods were a superior live prey when compared to rotifers in first feeding ABT larvae, as indicated by the higher growth achieved by copepod-fed larvae, possibly reflecting the higher protein content of the copepods (Betancor et al., 2017b). However, the mass production of *Acartia* copepods its labourious and costly in comparison to the production of enriched rotifers or *Artemia*, which explains why copepods are not widely used as a live prey in hatcheries for any species of marine fish.

In the present study, further feeding trials were performed to expand our knowledge of early nutrition and nutritional requirements of first feeding ABT larvae. Our overarching aim was to evaluate different strategies during first feeding (up to weaning onto an artificial, formulated diet) of ABT larvae from a performance, compositional and molecular perspective. In order to do so, two groups of ABT larvae were fed with either copepod (*Acartia tonsa*; C) nauplii, or rotifers (*Brachionus rotundiformis*; R) enriched with Algamac 3050® from mouth opening to 13 dah. After this, the C-larvae group was fed either *Artemia* enriched with Algamac 3050® (CA), *Acartia* nauplii and copepodites (CC) or sea bream (*Sparus aurata*) yolk-sac larvae (CY), while the R-larvae group were fed on *Artermia* enriched with Algamac 3050® (RA) up to 18 dah. Growth performance, developmental indices and survival were determined. Additionally, the expression of genes related to lipid metabolism (transcription factors, fatty acid metabolism and lipid homeostasis), antioxidant enzymes, myogenesis and digestive enzymes was carried out in order to assess the impacts of the different feeding protocols on larval metabolism and physiology.

2. Materials and methods

2.1. Atlantic bluefin tuna larvae rearing conditions

The ABT eggs used in this study were obtained in June 2017 from ABT broodstock fish maintained in captivity in a floating net cage located at El Gorguel, off the Cartagena coast, SE Spain. Captive-reared ABT broodstock fish spawned naturally and spontaneously, and floating eggs were collected inside the cage by means of a net of 500 μm mesh screen size. A 1.5 m polyvinyl sheet was also placed around the inside of the cage to avoid eggs drifting away from the cage (or into the cage) by means of currents and/or waves. Collected eggs were transported in a 500 L plastic tank supplied with pure oxygen to the Spanish Institute of Oceanography (IEO) Planta Experimental de Cultivos Marinos (Puerto de Mazarrón, Murcia, Spain) aquaculture facilities and placed in 100 L tanks with gentle oxygenation and flow-through sterilized seawater. After 1 h, aeration and water flow were stopped to separate buoyant (viable) from non-buoyant (non-viable) eggs. After washing and counting, the fertilized eggs were incubated in 1400 L cylindrical tanks at a density of 8.5 eggs L^{-1}. Incubation was carried out at a water temperature 23–25 °C, 37‰ salinity, dissolved oxygen 6.5 mg L^{-1} and continuous photoperiod, with a light intensity of 1000 lx. An upwelling flow-through with gentle aeration was employed in order to maintain oxygen levels near to saturation. Larvae hatched approximately 32 h after fertilization, with a hatching rate of almost 90%, and were fed with enriched rotifers (R-larvae) or copepod nauplii (C-larvae) from 2 dah to 13 dah. A mixture of the microalgae *Isochrysis* sp. (T-Iso) and *Chlorella* (V12 DHA-enriched, Pacific Trading Co., Japan) were added to tanks at a density of 2–3 × 10^5 cells mL^{-1} as green water. During the feeding trial, photoperiod was maintained at 14 h/10 h light/dark (light intensity about 500 lx), temperature ranged between 23 and 25 °C and daily water renewal was 100–200% tank volume day^{-1}. Incoming seawater was filtered at 10 μm and UV sterilized. An upwelling current was created to avoid larvae sinking (mainly at night) and to maintain oxygen level (Ortega, 2015; De La Gándara et al., 2016; Betancor et al., 2017a,b). The rearing conditions for feeding trials are summarized in Supplementary Table 1.

2.2. Dietary trial 1 with live prey, rotifers and copepod nauplii, from first feeding up to 13 dah

In the first trial, ABT larvae at a density 8.5 larvae L^{-1} were fed the two different live prey in quadruplicate (4 tank replicates/treatment) from mouth opening (2 dah) up to 13 dah (Supplementary Table 1). Larvae were fed either with rotifer *Brachionus rotundiformis* enriched with Algamac 3050® (treatment R) or *Acartia tonsa* copepod nauplii (treatment C). To maintain constant live prey concentration of 10 rotifer mL^{-1} or copepod nauplii/copepodite mL^{-1} within each experimental tank, three water samples (10 mL) from each tank were counted twice per day before supplying new feed (Ortega, 2015; De La Gándara et al., 2016; Betancor et al., 2017a,b).

2.3. Dietary trial 2 from 13 to 18 dah

After sampling ABT larvae at 13 dah, the remaining larvae were redistributed into four 1400 L tanks at a density of 0.43 larvae L^{-1} (Supplementary Table 1). The dietary treatments in single replicate tanks were established as follows: group C-larvae (ABT larvae that had been previously fed on *A. tonsa* nauplii) were given three different feeds, being either *Artemia* enriched with Algamac 3050® at 5 metanauplii mL^{-1} (treatment CA), *Acartia* nauplii and copepodites at 10 nauplii/copepodites mL^{-1} (treatment CC) or gilthead sea bream (*Sparus aurata*) yolk-sac larvae at 5 larvae mL^{-1} (treatment CY). The R group (ABT larvae that had been fed on rotifer *Brachionus rotundiformis* enriched with Algamac 3050®) were passed on to being fed only *Artermia* enriched with Algamac 3050® (treatment RA) at 5 metanauplii mL^{-1} to represent a reference treatment reflecting the common commercial protocol for marine fish larvae. All four groups were fed these treatments from 13 dah to 18 dah when larvae were again sampled.

2.4. Rotifer culture and enrichment protocol

S-type rotifers *B. rotundiformis* were continuously cultured with comercial DHA-enriched algal paste (Chlorella V-12; Chlorella Industry, Kyushu, Japan), at a concentration of 3 mL Chlorella paste per 10^6 rotifers per day, in four 2000 L cylindro-conical tanks supplied with filtered and sterilized sea water at 24–26 °C, 38‰ salinity, dissolved oxygen at saturation level and 24 h continual illumination. Enrichment treatment consisted of the commercial product, Algamac 3050®. Additionally, rotifers were supplemented with taurine (0.5 g per 10^6 rotifers), organic Se (Selplex® Alltech Spain SL; 3.0 mg per 10^6 rotifers), and vitamin E, as dl-α tocopheryl acetate (Lutavit E50; (BASF; 0.9 mg per 10^6 rotifers), 18 h before the enrichment treatment. The enrichment

protocols were performed in 100 L cylindro-conical tanks at a density of 1000 rotifers mL^{-1}, adding a dose of the enrichment product of 0.3 g plus 3 mg Selplex® and 0.9 mg vitamin E per 10^6 rotifers over a period of 6 h for Algamac 3050® according to manufacturer's recommendations.

2.5. Cultivation of the copepod Acartia tonsa

The copepods (*A. tonsa*) were cultivated in 4000 L cylindrical tanks with seawater of 34‰ salinity at 20 °C and were continuously fed with algae *Rhodomonas baltica* at a concentration not below 3×10^4 cells mL^{-1}. *Acartia* eggs were obtained every day with a harvesting arm to collect the eggs deposited on the flat bottom of the tanks. The eggs were washed thoroughly and stored in flasks at 2 °C. Egg harvest started 3 months in advance of the ABT feeding trial and continued until the end of the trial. The water in the flasks was renewed every 2 weeks and the number of eggs counted. The copepod eggs were incubated in 100 L tanks at a maximum density of 150 eggs mL^{-1}. From 2 days after hatch the nauplii and copepodites were fed *ad libitum* with a mixture of *R. baltica* and *Isochrysis galbana* clone T-Iso and, before harvesting, the nauplii/copepodite density in the tanks was estimated, harvested with a siphon, concentrated in a 60 μm sieve and then transferred to the ABT larval tanks.

2.6. Artemia metanauplii enrichment and gilthead sea bream yolk sac larvae

Artemia cysts EG type were decapsulated and enriched with Algamac 3050® over 12 h before being fed to ABT larvae. Recently hatched nauplii were disinfected with bronopol (100 ppm; (Pyceze®; Novartis). Enriched *Artemia* metanauplii were deposited in the tanks at a density of 0.1–0.2 metanauplii mL^{-1}. Before reproduction, gilthead sea bream broodstock were fed Vitalis CAL and Vitalis REPRO (Skretting®) at a rate of 0.6–0.8% of broodstock biomass. Gilthead sea bream 1 dah yolk sac larvae were added to tanks at a density of 5–10 larvae L^{-1}.

2.7. Sampling for biometrical, biochemical and molecular analysis

Thirty and twenty randomly caught ABT larvae per replicate treatment were anaesthetized (0.02% 2-phenoxyethanol, Sigma, Spain) in Trial 1 and 2, respectively, total length measured and individual larvae photographed. The tank was considered the experimental unit in Trial 1 (n = 4), where individual fish were utilized in Trial 2 (n = 20). The developmental stage was assessed by counting the number of ABT larva which had attained full flexion of the notochord by the end of the feeding trials (13 and 18 dah) in each replicate set of samples. Individual larvae dry mass was determined on a precision balance after maintaining samples at 110 °C for 24 h and cooling *in vacuo* for 1 h before weighing. Final survival (%) was calculated by counting individual live larvae at the beginning and end of the trial.

In trial 1, three samples of 15 larvae per sample of 13 dah larvae were collected per tank: i) two samples/tank were placed in 1 mL of RNA*later*® (Sigma, Madrid, Spain) for RNA extraction and molecular analysis (n = 8), and ii) one sample/tank was frozen in liquid N_2 and stored at −80 °C for biochemical analysis (n = 4). In trial 2, nine samples of 15 larvae of 18 dah larvae were collected per tank with 6 samples/tank used for molecular analysis (n = 6) and 3 samples/tank used for biochemical analyses, respectively (n = 3). Triplicate samples of enriched rotifers, copepods (*Acartia*), enriched *Artemia* metanauplii, and 1 dah sea bream yolk sac larvae were filtered and washed, excess water drained and blotted with filter paper, immediately frozen in liquid N_2 and stored at −80 °C prior to analysis. All procedures were carried out according to the current national and EU legislation on the handling of experimental animals.

2.8. Biochemical analysis

2.8.1. Proximate gross composition

Proximate compositions of live feeds (protein and lipid) were determined according to standard procedures (AOAC, 2000). Three technical replicates of feeds (single batch production) were freeze-dried prior to analyses. Ash content determined after incineration at 600 °C for 16 h. Crude protein was measured by determining nitrogen content (N × 6.25) using automated Kjeldahl analysis (Tecator Kjeltec Auto 1030 analyser, Foss, Warrington, UK), and crude lipid content determined gravimetrically after Soxhlet lipid extraction (Tecator Soxtec system 2050 Auto Extraction apparatus). Carbohydrate content was calculated as 100 – (percentages of protein + lipid based on dry weight). The gross energy content was calculated from gross composition data and using values of 5.65, 9.45 and 4.20 kcal g^{-1} for protein, lipid and carbohydrates, respectively (Henken et al., 1986).

2.8.2. Preparation of hydrolysates, derivatisation, UPLC analysis of taurine and total amino acid content of live prey and ABT larvae, and calculation of amino acid sufficiency index

A Waters AccQ-Tag Ultra Method® was used in determining taurine and amino acids contents in samples of enriched rotifers *B. rotundiformis* and 13 dah ABT larvae. Hydrolysis and derivatization were carried out according to manufacturer's instructions, and amino acid analysis (including taurine) was performed using a Waters H-Class UPLC fitted with an ACQUITY BEH Phenyl 1.7 μ UPLC column (AAA for H-Class System Guide, Waters Corporation 2012). An essential amino acid sufficiency index (SIa.a.) was calculated by assuming the total amino acid profile/content of 1 dah ABT yolk sac larvae as an indicator of ABT larvae amino acid requirements. The index was calculated by dividing the content of a determined amino acid in a live prey by the content of the same amino acid in 1 dah ABT larvae and multiplying the result by 100.

$$\text{SIa. a.} = ([\text{a. a.prey}]/[\text{a. a. 1 dah ABT larvae}]) \times 100.$$

Values of a SIa.a. equal or above 100 will indicate sufficient dietary amount of that amino acid in the live prey, whereas values below 100 show potential insufficiency for that amino acid (Oser, 1959).

2.8.3. Total lipid, lipid class composition and fatty acid analysis

Total lipid of live feeds (enriched rotifers, copepods, enriched *Artemia* and gilthead sea bream yolk sac larvae) and ABT larvae fed the different dietary regimes was extracted from triplicate pooled samples according to the method of Folch et al. (1957). Approximately 200 mg samples of feeds/ABT larvae were placed in 10 mL of ice-cold chloroform/methanol (2:1, by vol) and homogenized with an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, U.K.). The non-lipid and lipid layers were separated by addition of 5 mL of 0.88% (w/v) KCl and allowed to separate on ice for 1 h. The upper aqueous layer was aspirated and the lower organic layer dried under oxygen-free nitrogen. The lipid content was determined gravimetrically after drying overnight in a vacuum desiccator.

Lipid class composition was determined by high-performance thin-layer chromatography (HPTLC) using 10 × 10 cm plates (VWR, Lutterworth, England). Approximately 1 μg of total lipid was applied as a single spot, and the plates developed in methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol.) to two-thirds up the plate. After drying for 20 min, the plate was fully developed with isohexane/diethyl ether/acetic acid (85:15:1, by vol.). The lipid classes were visualized by charring at 160 °C for 15 min after spraying with 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid and quantified by densitometry using a CAMAG-3 TLC scanner (version Firmware 1.14.16). Scanned images were recorded automatically and analyzed using winCATS Planar Chromatography Manager software (version 1.2.0) (Henderson and Tocher, 1992).

Fatty acid methyl esters (FAME) from the extracted total lipids were prepared by acid-catalyzed transesterification at 50 °C for 16 h

according to the method of Christie (1993). Methyl esters were separated and quantified by gas-liquid chromatography (Agilent Technologies 7890B GC System) using a 30 m × 0.32 mm i.d. fused silica capillary column (SUPELCOWAX™-10, Supelco Inc., Bellefonte, USA) and on-column injection at 50 °C. Hydrogen was used as carrier gas and temperature programming was from 50 °C to 150 °C at 40 °C per min and then to 230 °C at 2.0 °C per min. Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman, 1980; Tocher and Harvie, 1988). Data were collected and processed using Agilent Technologies Openlab CDS Chemstation for Windows (version A.02.05.21).

2.8.4. Determination of alpha-tocopherol (vitamin E) content

Alpha-tocopherol concentrations in enriched rotifers and Artemia as well as copepods were determined using high-pressure liquid chromatography (HPLC) with UV detection. Samples were weighed, homogenized in pyrogallol, and saponified as described by McMurray et al. (1980) and Cowey et al. (1981). HPLC analysis was performed using a 150 × 4.60 mm, reverse-phase Luna 5 lm C18 column (Phenomenox, CA, USA). The mobile phase was 98% methanol pumped at 1.0 mL min^{-1}. The effluent from the column was monitored at a wavelength of 293 nm and quantification achieved by comparison with alpha-tocopherol (Sigma-Aldrich) as external standard.

2.8.5. Selenium determination

Total selenium concentration was measured in feeds according to the method established by Betancor et al. (2012). Dried samples were weighed in three replicates of between 0.04 and 0.1 g and digested in a microwave digester (MarsXpress, CEM, USA) with 5% of 69% pure nitric acid in three steps as follows; 21 °C to 190 °C for 10 min at 800 W, then 190 °C for 20 min at 800 W, and finally a 30 min cooling period. The digested solution was poured into a 10 mL volumetric flask and made up to volume with distilled water. A total of 0.4 mL of this solution was added to 10 mL tubes, 10 µL of internal standard (Gallium and Scandium, 10 ppm, BDH, UK) included and 0.2 mL of methanol added. The tube was made up to volume with distilled water and total selenium was measured in a reaction cell by Inductively Coupled Plasma Mass Spectrometry (Thermo Scientific, XSeries2 ICP-MS, USA), using argon and hydrogen as carrier gas.

2.8.6. Stable isotope analysis

Triplicate samples of rotifer *B. rotundiformis* enriched with Algamac 3050®, *A. tonsa* copepod nauplii and 13 dah ABT larvae fed on rotifers and/or copepods were frozen in liquid nitrogen and subsequently lyophilized. Then, samples were ground to powder by pestle and mortar, packed into tin capsules to be analyzed for isotopic relative abundance (δ) of ^{15}N and ^{13}C, carbon (%) and nitrogen (%). Prior to $\delta^{13}C$ analysis, samples with high lipid content (C:N ratio > 3.5; see Post et al., 2007) were subjected to total lipid extraction by chloroform/methanol (2:1, by volume) (Varela et al., 2012, 2013). The relative abundances of ^{13}C and ^{15}N ($\delta^{13}C$ and $\delta^{15}N$, repectively) were measured by a continuous gas flow system using a Thermo Finnigan Elementary Analyzer Flash EA1112 coupled to a Finnigan MAT Delta Plus mass spectrometer. All carbon and nitrogen isotope data are reported in δ notation according to the following equation: $\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000$, where X is ^{13}C or ^{15}N and R is the ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$ (Peterson and Fry, 1987). Standard materials are Vienna Pee Dee belemnite for carbon and atmospheric N_2 for nitrogen and expressed as parts per thousand (‰) relative to standards (Peterson and Fry, 1987). The isotopic enrichmen (Δ) of the predator in relation to its prey was calculated as: $\Delta^{15}N = \delta^{15}N_{predator} - \delta^{15}N_{prey}$ and $\Delta^{13}C = \delta^{13}C_{predator} - \delta^{13}C_{prey}$, respectively.

2.9. Tissue RNA extraction and cDNA synthesis

Samples of pooled larvae (approximately 100 mg) [2 samples per tank in Trial 1 (n = 8) and 6 samples per tank in trial 2 (n = 6)] were placed in RNA*later*® (Sigma–Aldrich, Dorset, UK) and frozen at −20 °C prior to total RNA extraction. Samples were homogenized in 1 mL of TriReagent® (Sigma-Aldrich) RNA extraction buffer using a bead tissue disruptor (Bio Spec, Bartlesville, Oklahoma, USA). Total RNA was isolated following manufacturer's instructions and quantity and quality determined by spectrophotometry using a Nanodrop ND-1000 (Labtech Int., East Sussex, UK) and electrophoresis using 200 ng of total RNA in a 1% agarose gel. cDNA was synthesized using 2 µg of total RNA and random primers in 20 µL reactions and the high capacity reverse transcription kit without RNase inhibitor according to the manufacturer's protocol (Applied Biosystems, Warrington, UK).

2.10. qPCR analysis

Several genes related to lipid and fatty acid metabolism, antioxidant and digestive enzymes, as well as growth markers were evaluated in the present study. Quantitative real-time PCR (qPCR) was carried out on transcription factors peroxisome proliferator-activated receptor alpha (*ppar*α), peroxisome proliferator-activated receptor gamma (*ppar*γ), liver X receptor (*lxr*), retinoid X receptor (*rxr*), sterol regulatory element-binding protein 1 (*srebp1*) and sterol regulatory element-binding protein 2 (*srebp2*); LC-PUFA biosynthesis genes delta-6 fatty acyl desaturase (*fads2d6*) and fatty acyl elongase 5 (*elovl5*), and fatty acid metabolism genes fatty acid synthase (*fas*), carnitine palmitoyl transferase I (*cpt1*), acyl coA oxidase (*aco*), fatty acid binding protein 2 (intestinal; *fabp2*), fatty acid binding protein 4 (adipocyte; *fabp4*), fatty acid binding protein 7 (brain; *fabp7*), lipoprotein lipase (*lpl*) and 3-hydroxy-3-methylglutaryl-CoA lyase (*hmgcl*); the antioxidant enzymes superoxide dismutase (*sod*), catalase (*cat*), glutathione peroxidase 1 (*gpx1*) and glutathione peroxidase 4 (*gpx4*); myogenesis growth indicators myosin heavy chain (*myhc*) and tropomyosin (*tropo*); and digestive genes trypsin (*tryp*), amino peptidase (*anpep*), alkaline phosphatase (*alp*), amylase (*amy*), pancreatic lipase (*pl*), phospholipase A2 (*pla2*), bile salt activated lipase 1 (*bal1*) and bile salt activated lipase 2 (*bal2*) (see Supplementary Table 2).

Expression of genes of interest was determined by qPCR of all the RNA samples with *Elongation factor-1α* (*elf1α*) and *β-actin* used as reference genes. The cDNA was diluted 20-fold with milliQ water. The efficiency of the primers for each gene was previously evaluated by serial dilutions of cDNA pooled from the samples to guarantee it was > 85% for all primer pairs. qPCR was performed using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates in duplicate 20 µL reaction volumes containing 10 µL of Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific, Hemel Hempstead, UK), 1 µL of the primer corresponding to the analyzed gene (10 pmol), 3 µL of molecular biology grade water and 5 µL of cDNA (1/20 diluted). In addition, amplifications were carried out with a systematic negative control (NTC, no template control) containing no cDNA. Standard amplification parameters contained an UDG pretreatment at 50 °C for 2 min, an initial denaturation step at 95 °C for 10 min, followed by 35 cycles: 15 s at 95 °C, 30 s at the annealing Tm and 30 s at 72 °C.

2.11. Statistical analysis

Results for biometry, lipid class and fatty acid compositions are presented as means ± SD (n = 20 for biometry and n = 3 for survival, lipid class and fatty acid compositions). The data were checked for homogeneity of the variances by the Bartlett test and, where necessary, arc-sin transformed before further statistical analysis. Relations between dietary components and the different variables measured were surveyed by correlation and linear regression analysis. Differences between mean values were analyzed by *t*-test and one-way ANOVA followed by Tukey's multiple comparisons test performed using GraphPad Prism version 7.00 for Mac OSX (GraphPad Software, La Jolla

Table 1
Total gross composition (% dry mass), energy content (kcal g^{-1}), taurine (mg g^{-1}), vitamin E (μg g^{-1}dry mass), Se (μg g^{-1}dry mass) and lipid class composition (total lipid %) of rotifer *Brachionus rotundiformis* (ROT) enriched with Algamac 3050®, nauplii of the copepod *Acartia tonsa* (COP) fed with the microalgae *Rhodomonas baltica*, *Artemia* metanauplii (ART) enriched with Algamac 3050®, and gilthead sea bream (*Sparus aurata* L.) yolk sac larvae (YSL) used to feed Atlantic bluefin tuna (*Thunnus thynnus* L.) larvae.

	ROT	COP	ART	YSL
Composition (% dry mass)				
Protein	51.3 ± 0.4^{c}	63.2 ± 2.1^{a}	53.8 ± 0.8^{c}	58,7 ± 0,4^{b}
Lipid	12.0 ± 0.5^{b}	8.8 ± 0.3^{c}	18.1 ± 0.9^{a}	18.9 ± 0.4^{a}
Carbohydrate	30.1 ± 0.6^{a}	20.4 ± 1.1^{b}	20.0 ± 0.7^{b}	13.7 ± 0.3^{c}
Ash	6.7 ± 0.3^{c}	7.6 ± 0.3^{b}	8.1 ± 0.1^{a}	8.7 ± 0.2^{a}
Energy (kcal·g^{-1})				
Protein	28.6 ± 0.7^{c}	35.3 ± 0.9^{a}	30.2 ± 0.6^{c}	33.0 ± 0.4^{b}
Lipid	11.3 ± 0.4^{b}	8.5 ± 0.3^{c}	16.9 ± 0.7^{a}	17.9 ± 0.6^{a}
Carbohydrate	12.6 ± 0.3^{a}	8.4 ± 0.3^{b}	8.4 ± 0.5^{b}	5.9 ± 0.3^{c}
Total energy	52.5 ± 0.6^{b}	52.2 ± 0.7^{b}	55.5 ± 0.6^{a}	56.8 ± 0.5^{a}
Taurine (mg·g^{-1})	2.5 ± 0.2^{d}	4.1 ± 0.2^{c}	6.1 ± 0.2^{b}	11.0 ± 0.8^{a}
Vitamin E (μg·g^{-1})	232.3 ± 1.7^{b}	170.1 ± 6.5^{c}	78.9 ± 5.8^{d}	308.7 ± 4.6^{a}
Se (μg·g^{-1})	4.8 ± 0.2^{a}	0.4 ± 0.0^{c}	1.8 ± 0.5^{b}	1.4 ± 0.1^{b}
Lipid classes (% total lipid)				
PC	12.3 ± 0.3^{c}	16.2 ± 0.3^{b}	9.2 ± 0.2^{d}	18.7 ± 1.0^{a}
PE	13.1 ± 1.1^{b}	11.8 ± 0.2^{b}	7.7 ± 0.2^{c}	17.1 ± 0.4^{a}
TPL	41.0 ± 0.3^{c}	52.9 ± 0.7^{a}	28.6 ± 1.0^{d}	47.9 ± 0.5^{b}
Cholesterol	6.6 ± 0.3^{c}	11.9 ± 0.7^{b}	11.8 ± 0.4^{b}	21.7 ± 0.8^{a}
Triacylglycerol	40.2 ± 0.3^{b}	23.3 ± 0.3^{c}	46.3 ± 0.5^{a}	10.0 ± 0.7^{d}
TNL	59.0 ± 0.3^{b}	47.1 ± 0.7^{d}	71.4 ± 1.0^{a}	52.1 ± 0.5^{c}
TPL/TNL	0.7 ± 0.1^{ab}	1.1 ± 0.2^{a}	0.4 ± 0.1^{b}	0.9 ± 0.2^{a}

Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. Values bearing different superscript letter are significantly different (P < .05). TNL, total neutral lipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TPL, total polar lipids.

California USA, www.graphpad.com). Differences were reported as statistically significant when P < .05 (Zar, 1999). Gene expression results were analyzed using the relative expression software tool (REST 2009), which employs a pairwise fixed reallocation randomization test (10,000 randomizations) with efficiency correction (Pfaffl et al., 2002) to determine the statistical significance of expression ratios (gene expression fold changes) between two treatments.

3. Results

3.1. Analyzed composition of the different dietary treatments (rotifer, Artemia, copepods and gilthead sea bream yolk sac larvae)

Gross composition (% dry mass), energy content (kcal·g^{-1} dry mass), taurine (mg g^{-1} dry mass), vitamin E and selenium contents (μg g^{-1} dry mass) and lipid class composition (total lipid %) of the different live preys used in the present study are shown in Table 1. Total protein content was significantly highest in *Acartia* copepod (63.2%) followed by sea bream yolk sac larvae (58.7%) and then enriched rotifer and *Artemia* (about 52%). In contrast, total lipid content was highest in sea bream yolk sac larvae and enriched *Artemia* (about 18%), followed by enriched rotifer (12%) and copepod (8.8%). Carbohydrates (generally mostly chitin) was highest in enriched rotifers, followed by *Acartia* and *Artemia* and, as expected, lowest in sea bream yolk sac larvae. Total caloric content was significantly highest in sea bream yolk sac larvae and enriched *Artemia* (about 56 kcal g^{-1}), due to the highest lipid caloric contribution, followed by enriched rotifers and *Acartia* (about 52 kcal g^{-1}). The significantly highest total polar lipid contents and polar/neutral lipid ratios were found in *Acartia* nauplii and sea bream yolk sac larvae, mainly due to presenting highest levels of PC and PE. Enriched *Artemia* and rotifer had the significantly lowest polar/neutral lipid ratios, due to their high triacylglycerol (TAG) contents. Free chlolesterol was highest sea bream yolk sac larvae > *Acartia* nauplii > enriched *Artemia* metanuplii > rotifers. Vitamin E content was highest in sea bream yolk sac larvae, followed by enriched rotifers, *Acartia* nauplii and lastly enriched *Artemia*. Se content was highest in enriched rotifers followed by enriched *Artemia* and yolk sac sea bream larvae and then *Acartia* copepods. Highest taurine content was found in gilthead sea bream yolk sac larvae (11 mg g^{-1} dry mass), followed by enriched *Artemia* (6.1 mg g^{-1} dry mass), *Acartia* nauplii (4.1 mg g^{-1} dry mass) and finally enriched rotifer (2.5 mg g^{-1} dry mass).

3.2. Amino acid contents in live preys and ABT larvae

Highest total amino acid content was presented by *Acartia* nauplii (571.5 mg g^{-1} dry mass) > gilthead sea bream yolk sac larvae (528.3 mg g^{-1} dry mass) > enriched *Artemia* metanauplii (411.6 mg g^{-1} dry mass) > enriched rotifer (334.3 mg g^{-1} dry mass) (Table 1 and Supplementary Table 3). However, the highest total essential amino acid content was shown by sea bream yolk sac larvae (280.4 mg g^{-1} dry mass) > *Acartia* nauplii (267.1 mg g^{-1} dry mass) > enriched *Artemia* metanauplii (214.6 mg g^{-1} dry mass) > enriched rotifer (157.5 mg g^{-1} dry mass). The most deficient content of essential amino acids, according to the sufficiency indices, was presented by enriched rotifers (Table 2). Enriched *Artemia* showed some insufficiencies with regards to valine, leucine, phenylalanine, threonine and methionine. *Acartia* nauplii showed shortfall of histidine, and methionine, whereas sea bream yolk sac larvae only showed a slight shortage of valine.

3.3. Total lipid fatty acid compositions and contents of the live preys used to feed ABT larvae in Trials 1 and 2

Total lipid fatty acid composition (percentage of weight) of rotifer *B. rotundiformis* enriched with Algamac 3050®, nauplii of the copepod *A. tonsa* fed with the microalgae *R. baltica*, *Artemia* metanauplii enriched with Algamac 3050® and gilthead sea bream (*S. aurata* L.) yolk sac larvae used to feed ABT larvae are presented in Table 3. The highest values of total saturated fatty acids were shown by *Acartia* nauplii and gilthead sea bream yolk sac larvae (27.8% and 26.0%, respectively), followed by enriched rotifer (23.9%) and *Artemia* (22.4%). The highest values for total monoenes were presented by enriched *Artemia* and yolk sac larvae (27.5% and 26.3%, respectively), mainly due to the large proportions of 18:1n-9 (14.9% and 15.9%, respectively). Copepods and

enriched rotifers showed monoene values significantly lower (11.5% and 12.6%, respectively). Total n-6 PUFA were highest in enriched rotifers, followed by *Acartia* nauplii, enriched *Artemia* and yolk sac larvae, largely due to their contents of linoleic acid (18:2n-6, LA). Yolk-sac larvae showed a very low level of arachidonic acid (20:4n-3, ARA) compared to the other live preys. Total n-3 PUFA were highest in copepod nauplii (43.9%) > enriched *Artemia* and yolk sac larvae (39.3% and 37.2%, respectively) > enriched rotifers (32.9%). Enriched *Artemia* presented the significantly highest value (23.0%) of linolenic acid (18:3n-3; LNA) characteristic of EG grade *Artemia*. The proportion of eicosapentaenoic acid (20:5n-3, EPA) was significantly highest (7.2%) and that of docosahexaenoic acid (22:6n-3, DHA) second highest (25.1%) in yolk sac larvae, whereas the proportion of docosahexaenoic acid was highest in *Acartia* (27.0%). The DHA/EPA ratio was highest in *Acartia* (6.1), followed by yolk sac larvae (3.5), enriched rotifers (2.8) and finally enriched *Artemia* (1.9), respectively (Table 3). Quantitative results as total lipid fatty acid content (μg fatty acid mg dry mass^{-1}) of the live preys used to feed ABT larvae in Trials 1 and 2 are shown in Supplementary Table 4.

3.4. Growth performance and survival rates of ABT larvae in Trial 1 (13 dah) and Trial 2 (18 dah)

Growth performance and survival of ABT larvae in Trial 1 (13 dah) and Trial 2 (18 dah) are presented in Table 4. In Trial 1, 13 dah ABT larvae fed on *Acartia* copepods showed significantly higher values for total length, total weight and flexion index than larvae fed on enriched rotifers. However, no significant differences were observed in survival rates. In Trial 2, 18 dah ABT larvae, previously fed on copepods in Trial 1 and then on sea bream yolk sac larvae (treatment CY), showed the significantly highest values for total length, total weight and survival (32%), and second lowest for dry mass %. The next best set of values for growth and survival were obtained for ABT larvae fed on treatment CA, followed by those of larvae fed on treatment CC, with poorest performance obtained with ABT larvae fed treatment RA.

3.5. Total lipid content and lipid class composition of 13 dah and 18 dah ABT larvae under different live prey feeding protocols in Trials 1 and 2

Total lipid content (% of live and dry mass) and lipid class composition (total lipid %) of ABT larvae 13 dah fed on treatments R and C in Trial 1, and ABT larvae 18 dah that had been fed on treatments RA, CA, CC and CY in Trial 2, are presented in Table 5. In Trial 1, ABT 13 dah larvae fed either on treatment R or C did not show significant differences in total lipid content. Neither showed significant differences in total polar or total neutral lipids. Nevertheless, total polar lipids predominated (56.5 and 58.5%, respectively) over total neutral lipids (43.5 and 41.5%, respectively) in ABT larvae fed on treatments R or C, respectively. Moreover, ABT larvae fed on treatment R showed significant higher level of TAG than larvae fed on treatment C (14.5% *vs* 10.7%). In Trial 2, ABT 18 dah fed on treatments CY and RA showed significant highest lipid contents followed by those ABT larvae that were fed on treatments CC and *CA*. Total polar lipid showed highest (59.4%) in ABT larvae from treatment CC, followed by ABT larvae from treatments CA and RA (56.4% and 54.6%, respectively) and lowest value for larvae from CY treatment, due to their highest level of total neutral lipid (51.8%) primarily TAG (22.4%) (Table 5).

Table 2
Sufficiency Index (SI) of essential amino acids (Oser, 1959) of rotifer *Brachionus rotundiformis* (ROT) enriched with Algamac 3050®, nauplii of the copepod *Acartia tonsa* (COP) fed with the microalgae *Rhodomonas baltica*, *Artemia* metenauplii (ART) enriched with Algamac 3050® and gilthead sea bream (*Sparus aurata* L.) yolk sac larvae (YSL) used to feed Atlantic bluefin tuna (*Thunnus thynnus* L.) larvae in Trials 1 and 2.

	ROT	COP	ART	YSL
Taurine	60.5	99.3	147.7	266.3
Valine	68.0	107.9	80.2	97.2
Isoleucine	78.4	970.1	835.8	947.8
Leucine	73.3	101.5	79.3	110.7
Phenylalanine	87.4	116.1	91.4	114.3
Histidine	54.1	93.7	74.6	121.9
Lysine	74.6	113.8	100.2	124.4
Arginine	79.4	127.6	110.4	127.6
Threonine	69.3	121.6	79.6	115.0
Methionine	48.5	95.9	63.5	101.1

Results are means (n = 3). Values equal or above 100 indicate sufficient amount of that amino acid in the live prey, whereas values below 100 show insufficiency for that amino acid.

Table 3
Total lipid fatty acid composition (weight %) of rotifer *Brachionus rotundiformis* (ROT) enriched with Algamac 3050®, nauplii of the copepod *Acartia tonsa* (COP) fed with the microalgae *Rhodomonas baltica*, *Artemia* metenauplii (ART) enriched with Algamac 3050 and gilthead sea bream (*Sparus aurata* L.) yolk sac larvae (YSL) used to feed Atlantic bluefin tuna (*Thunnus thynnus* L.) larvae.

Fatty acid	ROT	COP	ART	YSL
14:0	2.3 ± 0.1^{b}	9.8 ± 0.3^{a}	1.5 ± 0.1^{c}	2.1 ± 0.2^{b}
16:0	15.1 ± 0.4^{b}	13.0 ± 0.3^{c}	14.9 ± 0.3^{b}	18.7 ± 0.3^{a}
18:0	4.8 ± 0.2^{a}	3.0 ± 0.1^{b}	5.1 ± 0.1^{a}	4.6 ± 0.4^{ab}
Total saturated[1]	23.9 ± 0.3^{b}	27.8 ± 0.8^{a}	22.4 ± 0.4^{b}	26.0 ± 0.4^{a}
16:1n-7	1.9 ± 0.2^{c}	3.3 ± 0.2^{b}	2.3 ± 0.1^{c}	4.8 ± 0.2^{a}
18:1n-9	2.2 ± 0.1^{c}	4.2 ± 0.1^{b}	17.2 ± 0.2^{a}	15.9 ± 0.4^{a}
18:1n-7	1.6 ± 0.2^{c}	2.0 ± 0.1^{bc}	6.5 ± 0.1^{a}	2.8 ± 0.2^{b}
20:1n-9	0.9 ± 0.2^{a}	0.3 ± 0.0^{c}	0.6 ± 0.1^{a}	0.6 ± 0.1^{a}
Total monoenes[2]	12.6 ± 0.4^{b}	11.5 ± 0.3^{b}	27.5 ± 0.6^{a}	26.3 ± 0.4^{a}
C16 PUFA	6.3 ± 0.3^{a}	3.1 ± 0.1^{b}	0.8 ± 0.2^{c}	0.9 ± 0.2^{c}
18:2n-6	16.3 ± 0.7^{a}	5.1 ± 0.3^{c}	4.3 ± 0.1^{d}	7.1 ± 0.3^{b}
20:4n-6	1.1 ± 0.1^{c}	1.4 ± 0.1^{b}	1.8 ± 0.2^{a}	1.6 ± 0.2^{ab}
22:5n-6	2.9 ± 0.4^{a}	3.3 ± 0.2^{a}	3.2 ± 0.2^{a}	0.3 ± 0.1^{b}
Total n-6PUFA[3]	24.8 ± 0.7^{a}	13.6 ± 0.3^{b}	10.0 ± 0.3^{bc}	9.7 ± 0.6^{c}
18:3n-3	3.9 ± 0.2^{b}	4.7 ± 0.2^{b}	23.0 ± 0.2^{a}	0.9 ± 0.1^{c}
18:4n-3	0.2 ± 0.0^{c}	4.8 ± 0.3^{a}	2.8 ± 0.1^{b}	0.6 ± 0.1^{c}
20:4n-3	1.0 ± 0.1^{a}	0.6 ± 0.1^{b}	0.6 ± 0.1^{b}	0.6 ± 0.1^{b}
20:5n-3	5.1 ± 0.3^{b}	4.4 ± 0.3^{bc}	4.1 ± 0.3^{c}	7.2 ± 0.2^{a}
22:5n-3	3.2 ± 0.4^{a}	0.2 ± 0.0^{b}	0.2 ± 0.0^{b}	2.6 ± 0.2^{a}
22:6n-3	14.1 ± 0.6^{b}	27.0 ± 0.9^{a}	8.0 ± 0.2^{c}	25.1 ± 0.9^{a}
Total n-3PUFA[4]	32.9 ± 0.9^{c}	43.9 ± 1.6^{a}	39.3 ± 0.3^{b}	37.2 ± 0.8^{b}
Total PUFA	57.7 ± 1.6^{a}	57.6 ± 1.4^{a}	50.1 ± 0.5^{b}	47.7 ± 0.9^{b}
n-3/n-6	1.3 ± 0.1^{c}	3.2 ± 0.2^{b}	3.9 ± 0.1^{a}	3.8 ± 0.2^{a}
DHA/EPA	2.8 ± 0.3^{c}	6.1 ± 0.7^{a}	1.9 ± 0.1^{d}	3.5 ± 0.2^{b}

Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. Values bearing different superscript letters are significantly different (P < .05). DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid.
[1] Totals include 15:0, 20:0, 22:0 and 24:0.
[2] Totals include 16:1n-9, 18:1n-11, 20:1n-7, 22:1 isomers and 24:1.
[3] Totals include 18:3n-6, 20:2n-6 and 22:4n-6.
[4] Totals include 20:3n-3 and 22:3n-3.

3.6. Total lipid fatty acid composition of 13 dah and 18 dah ABT larvae under different live prey feeding protocols in Trials 1 and 2

In Trial 1, 13 dah ABT larvae fed treatments R or C showed no significant differences between total saturated fatty acids, although larvae from treatment R showed significantly higher 16:0 contents than larvae from treatment C (Table 6). Total monoenes were significantly higher in larvae from treatment R, mainly due to higher levels of 16:1n-7 and 18:1n-7 in total lipids compared to larvae fed treatment C. The same trend was observed for total n-6 PUFA, which were significantly higher in ABT larvae fed treatment R, due to higher values of LA, ARA and n-6 docosapentaenoic acid (22:5n-6, DPA) compaed to larvae fed treatment C. In contrast, total n-3 PUFA content was highest in ABT larvae fed treatment C (34.2% *vs* 19.5%), mainly due to contain higher levels of LNA, SDA, EPA and DHA. The DHA/EPA ratio was almost 14-

Table 4
Growth performance and survival of ABT larvae in Trial 1 (13 dah) and Trial 2 (18 dah). Total length (mm), total weight as live mass or dry mass per larvae (mg), dry mass (%) and survival rate (%) of ABT larvae 13 days after hatch fed on rotifer *B. rotundiformis* enriched with Algamac 3050® (treatment R) or *A. tonsa* copepod nauplii (treatment C), and ABT larvae 18 days after hatch that were fed rotifer *B. rotundiformis* and then *Artemia* metanauplii enriched with Algamac 3050® (RA), *A. tonsa* copepod nauplii and then *Artemia* enriched with Algamac 3050® (treatment CA), *A. tonsa* copepod nauplii and then followed with *A. tonsa* nauplii and copepodites (treatment CC), and *A. tonsa* nauplii and then gilthead sea bream (*S. aurata*) yolk sac larvae (treatment CY).

Dietary treatments	Trial 1(13 dah ABT larvae)			Trial 2 (18 dah ABT larvae)		
	R	C	RA	CA	CC	CY
Total length (mm)	6.8 ± 0.3	7.6 ± 0.4*	8.2 ± 0.4^{b}	9.7 ± 0.5^{ab}	8.9 ± 0.6^{b}	10.3 ± 0.5^{a}
Total weight (mg larvae live mass^{-1})	4.3 ± 0.2	8.2 ± 0.5*	9.7 ± 0.5^{c}	11.6 ± 0.5^{b}	11.4 ± 0.6^{b}	24.3 ± 0.9^{a}
Total weight (mg larvae dry mass^{-1})	0.6 ± 0.1	1.1 ± 0.2*	1.2 ± 0.3^{c}	2.5 ± 0.4^{b}	1.9 ± 0.3^{bc}	3.6 ± 0.4^{a}
Dry mass (%)	14.4 ± 0.8	12.8 ± 0.9	12.2 ± 0.7^{d}	21.2 ± 1.1^{a}	16.9 ± 0.9^{b}	14.9 ± 0.8^{c}
Flexion Index (%)	35.8 ± 3.9	54.5 ± 5.0*	100	100	100	100
Survival at 13 dah (%)	13.9 ± 11.8	8.2 ± 3.8				
Survival at 18 dah (%)			2.4	2.1	2.3	3.6
Survival (13–18 dah, 5 days) (%)			17.6	19.5	21.7	32.0

Results are means ± SD (n = 4 in Trial 1 and n = 20 in Trial 2 for total length, total weight, dry mass and flexion index; n = 4 per treatment for survival in Trial A). An SD of 0.0 implies an SD of < 0.05. Values bearing an asterisk (*) between samples of 13 dah ABT larvae are significantly different (P < .05). Values of 18 dah ABT larvae bearing different superscript letters are significantly different (P < .05).

fold higher in ABT larvae fed treatment C.

In Trial 2, larvae fed treatments CC and CY showed significantly higher total saturated fatty acids, mainly due to higher levels of 16:0, followed by ABT larvae of the RA and CA treatments. Similarly, high levels of total monoenoic fatty acids (about 20% of total fatty acids, with half represented by 18:1n-9) were found in larvae from treatments RA, CA and CY, with larvae from treatment CC showing lower levels. Total n-6 PUFA were highest in larvae from treatment RA (14.9%) > larvae fed treatments CC and CA (12.5% and 12.1%, respectively), and were lowest in larvae fed treatment CY (9.8%). These values mainly reflected the level of 18:2n-6 that followed the same order per treatment. ARA was significantly highest in larvae fed treatment CA (1.9%), with no significant differences among the other treatments. Total n-3 PUFA was significantly higher in ABT larvae fed treatment CC (33.2%) > CA, CY and RA (30.9%, 30.7% and 28.2%, respectively). These values reflected the major contribution of DHA, which was highest in larvae fed treatments CC and CY (24.1% and 22.2%, respectively), followed by larvae fed treatments CA and RA (15.8% and 10%). Levels of EPA in ABT larvae showed, in decreasing order of abundance, treatments RA and CY (4.7% and 4.4%, respectively, and not significantly different) followed by treatments CA and CC (3.4% and 2.1%, respectively). The DHA/EPA ratio was significantly highest in 18 dah ABT larvae fed treatment CC (11.3), followed by-larvae fed treatments CY (5.1), CA (4.6) and RA (2.1).

3.7. Relative abundance (δ) of stable isotopes ^{15}N and ^{13}C and C:N ratios of enriched rotifer B. rotundiformis, A. tonsa nauplii and 13 dah ABT larvae

The relative abundance (*δ*) of ^{15}N and ^{13}C (‰) and C:N ratio of rotifer *B. rotundiformis* enriched with Algamac 3050®, nauplii of the copepod *A. tonsa* fed on the microalgae *R. baltica* and ABT larvae 13 dah fed on *B. rotundiformis* and *A. tonsa* nauplii, as well as isotopic

Table 5
Total lipid content (% of live and dry mass) and lipid class composition (total lipid %) of Atlantic bluefin tuna (*Thunnus thynnus* L.) larvae (ABT) 13 days after hatch fed with rotifer *Brachionus rotundiformis* enriched with Algamac 3050® (R), *Acartia tonsa* copepod nauplii (C), and ABT larvae 18 days after hatch that had been fed with rotifer *B. rotundiformis* and then *Artemia* metanauplii enriched with Algamac 3050® (RA), *Acartia* copepod nauplii and then *Artemia* enriched with Algamac 3050 ® (CA), *Acartia* copepod nauplii and then followed with *Acartia* nauplii and copepodites (CC), and *Acartia* nauplii and then gilthead sea bream (*Sparus aurata* L.) yolk sac larvae (CY).

Dietary treatments	13 dah ABT larvae			18 dah ABT larvae		
	R	C	RA	CA	CC	CY
Total lipid (% live mass)	1.3 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.7 ± 0.2	1.5 ± 0.2	1.8 ± 0.3
Total lipid (% dry mass)	9.1 ± 0.6	8.0 ± 0.7	10.5 ± 0.9^{a}	7.4 ± 0.6^{b}	8.3 ± 0.5^{b}	12.1 ± 0.9^{a}
Lipid classes (total lipid %)						
Phosphatidylcholine	21.2 ± 1.1	22.2 ± 1.2	21.6 ± 1.0	21.6 ± 01.4	23.2 ± 0.7	20.6 ± 0.9
Phosphatidylethanolamine	14.5 ± 1.2	14.6 ± 0.8	14.7 ± 0.8^{a}	14.0 ± 0.5^{a}	14.6 ± 0.3^{a}	11.4 ± 0.3^{b}
Phosphatidylserine	6.6 ± 0.2	7.2 ± 0.7	5.8 ± 0.4^{b}	6.5 ± 0.3^{ab}	7.8 ± 0.4^{a}	4.8 ± 0.2^{c}
Phosphatidylinositol	4.6 ± 0.3	3.7 ± 0.2*	4.2 ± 0.6	4.5 ± 0.5	4.6 ± 0.2	4.0 ± 0.5
Sphingomyelin	2.2 ± 0.1	3.8 ± 0.4*	2.1 ± 0.1^{c}	3.3 ± 0.1^{b}	4.0 ± 0.2^{a}	2.5 ± 0.2^{bc}
Phosphatidic acid/cardiolipin	3.3 ± 0.2	3.3 ± 0.1	3.2 ± 0.3^{a}	2.9 ± 0.7^{ab}	2.5 ± 0.2^{b}	2.5 ± 0.3^{b}
Lysophosphatidylcholine	0.4 ± 0.1	0.6 ± 0.2	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.0
Pigmented material	3 0.6 ± 0.8	3.1 ± 0.3	2.6 ± 0.2^{ab}	3.3 ± 0.5^{a}	2.5 ± 0.3^{ab}	2.0 ± 0.1^{b}
Total polar lipids	56.5 ± 1.7	58.5 ± 3.4	54.6 ± 2.1^{b}	56.4 ± 1.8^{b}	59.4 ± 0.6^{a}	48.2 ± 3.2b^{c}
Cholesterol/sterols	18.1 ± 0.6	17.5 ± 1.4	16.4 ± 1.1^{ab}	16.1 ± 1.8^{ab}	18.5 ± 0.6^{a}	14.6 ± 2.1^{b}
Free fatty acids	4.9 ± 1.1	6.2 ± 0.1	5.6 ± 0.7	5.1 ± 1.1	6.0 ± 0.6	5.7 ± 0.8
Diacylglycerol	2.1 ± 0.1	1.8 ± 0.2	1.4 ± 0.1^{b}	1.8 ± 0.5^{ab}	2.0 ± 0.3^{a}	1.9 ± 0.2^{a}
Triacylglycerol	14.5 ± 0.7	10.7 ± 1.0*	19.8 ± 1.2^{a}	15.4 ± 0.5^{b}	11.5 ± 0.3^{c}	22.4 ± 1.2^{a}
Wax/Sterol esters	3.9 ± 1.0	5.3 ± 0.7*	2.2 ± 0.4^{c}	5.4 ± 1.3^{b}	2.7 ± 0.4^{c}	7.2 ± 0.9^{a}
Total neutral lipids	43.5 ± 1.7	41.5 ± 2.4	45.4 ± 1.1^{b}	43.7 ± 1.7^{bc}	40.6 ± 0.8^{c}	51.8 ± 2.3^{a}

Results are means ± SD (n = 4 in Trial 1 and n = 3 in Trial 2). An SD of 0.0 implies an SD of < 0.05. Values bearing an asterisk (*) between samples of 13 dah ABT larvae (R and C) are significantly different (P < .05). Values of 18 dah ABT larvae (samples RA, CA, CC and CY) bearing different superscript letters are significantly different (P < .05).

Table 6
Total lipid fatty acid composition (weight %) of Atlantic bluefin tuna (*T. thynnus* L.) larvae 13 days after hatch fed with rotifer *B. rotundiformis* enriched with Algamac 3050 ® (R), *A. tonsa* copepod nauplii (C), and bluefin tuna larvae 18 days after hatch fed with rotifer *B. rotundiformis* and then *Artemia* metanauplii enriched with Algamac 3050® (RA), *A. tonsa* copepod nauplii and then *Artemia* enriched with Algamac 3050® (CA), *A. tonsa* copepod nauplii and then followed with *A. tonsa* nauplii and copepodites (CC), and *A. tonsa* nauplii and then gilthead sea bream (*S. aurata*) yolk sac larvae (CY).

Dietary treatments	13 dah ABT larvae			18 dah ABT larvae		
	R	C	RA	CA	CC	CY
Fatty acid (weight %)						
14:0	1.1 ± 0.2	1.4 ± 0.2	0.4 ± 0.1^{c}	0.8 ± 0.2^{b}	1.6 ± 0.2^{a}	0.4 ± 0.1^{c}
16:0	24.9 ± 0.9	21.2 ± 0.6*	17.7 ± 0.7^{b}	16.7 ± 0.5^{b}	20.5 ± 0.2^{a}	21.8 ± 0.3^{a}
18:0	11.4 ± 0.8	12.4 ± 0.4	12.9 ± 0.5^{a}	13.0 ± 0.4^{a}	11.4 ± 0.1^{b}	12.0 ± 0.2^{ab}
Total SFA[1]	39.5 ± 1.5	37.5 ± 1.2	33.7 ± 1.3^{b}	33.1 ± 0.3^{b}	36.9 ± 0.9^{a}	36.3 ± 0.5^{a}
16:1n-7	5.5 ± 0.2	2.2 ± 0.2*	1.8 ± 0.1^{b}	1.8 ± 0.1^{b}	2.4 ± 0.3^{a}	2.1 ± 0.2^{ab}
18:1n-9	6.8 ± 0.2	6.9 ± 0.2	10.9 ± 0.6^{b}	10.8 ± 0.2^{b}	7.5 ± 0.5^{c}	13.2 ± 0.8^{a}
18:1n-7	3.1 ± 0.2	2.1 ± 0.1*	4.8 ± 0.4^{a}	4.6 ± 0.5^{a}	2.0 ± 0.3^{c}	2.9 ± 0.3^{b}
20:1n-9	0.7 ± 0.2	1.1 ± 0.3	0.7 ± 0.1	0.4 ± 0.2	0.5 ± 0.1	0.6 ± 0.1
Total MUFA[2]	20.0 ± 0.6	14.0 ± 0.4*	20.2 ± 0.8^{a}	19.3 ± 0.4^{a}	14.2 ± 0.5^{b}	20.7 ± 1.4^{a}
C16 PUFA	2.1 ± 0.1	3.5 ± 0.5*	3.1 ± 0.2^{a}	3.0 ± 0.2^{a}	3.5 ± 0.3^{a}	2.1 ± 0.1^{b}
18:2n-6	4.5 ± 0.3	3.0 ± 0.1*	5.6 ± 0.3^{a}	4.8 ± 0.3^{ab}	4.6 ± 0.7^{ab}	4.1 ± 0.5^{b}
20:4n-6	5.1 ± 0.2	1.0 ± 0.1*	1.6 ± 0.1^{b}	1.9 ± 0.2^{a}	1.1 ± 0.0^{b}	1.2 ± 0.1^{b}
22:5n-6	4.3 ± 0.3	3.1 ± 0.2*	2.7 ± 0.2^{b}	3.0 ± 0.2^{ab}	3.6 ± 0.4^{a}	1.1 ± 0.1^{c}
Total n-6 PUFA[3]	17.5 ± 0.6	10.1 ± 0.8*	14.9 ± 0.6^{a}	12.1 ± 0.5^{b}	12.5 ± 0.3^{b}	9.8 ± 0.2^{c}
18:3n-3	0.3 ± 0.1	0.6 ± 0.1*	6.9 ± 0.3^{a}	5.7 ± 0.4^{b}	1.8 ± 0.3^{c}	0.4 ± 0.1^{d}
18:4n-3	0.5 ± 0.1	1.5 ± 0.3*	1.0 ± 0.2^{b}	1.8 ± 0.3^{a}	1.4 ± 0.3^{ab}	0.2 ± 0.0^{c}
20:4n-3	0.6 ± 0.1	0.6 ± 0.0	0.8 ± 0.2^{a}	0.8 ± 0.1^{a}	0.6 ± 0.1^{ab}	0.5 ± 0.1^{b}
20:5n-3	10.2 ± 0.8	3.1 ± 0.3*	4.7 ± 0.2^{a}	3.4 ± 0.3^{b}	2.1 ± 0.2^{c}	4.4 ± 0.2^{a}
22:5n-3	0.3 ± 0.0	0.4 ± 0.1	2.3 ± 0.4^{a}	0.3 ± 0.0^{b}	0.4 ± 0.1^{b}	1.8 ± 0.3^{a}
22:6n-3	5.9 ± 0.4	25.3 ± 0.9*	10.0 ± 0.4^{c}	15.8 ± 0.4^{b}	24.1 ± 1.0^{a}	22.2 ± 1.5^{a}
Total n-3 PUFA[4]	19.5 ± 0.6	34.2 ± 1.8*	28.2 ± 1.1^{b}	30.9 ± 1.4^{ab}	33.2 ± 1.3^{a}	30.7 ± 1.6^{ab}
Total PUFA	39.1 ± 1.7	47.5 ± 1.6*	43.1 ± 1.6^{ab}	43.0 ± 0.7^{ab}	45.7 ± 1.0^{a}	40.5 ± 1.6^{b}
DHA/EPA	0.6 ± 0.1	8.2 ± 0.4*	2.1 ± 0.1^{b}	4.6 ± 0.3^{a}	11.3 ± 0.7^{b}	5.1 ± 0.2^{a}
n-3/n-6	1.1 ± 0.2	3.4 ± 0.4*	1.9 ± 0.3^{c}	2.6 ± 0.2^{b}	2.6 ± 0.2^{b}	3.1 ± 0.2^{a}
Unknown	1.4 ± 0.2	1.0 ± 0.2	3.0 ± 0.3^{b}	4.6 ± 0.2^{a}	3.2 ± 0.4^{b}	2.5 ± 0.4^{c}

Results are means ± SD (n = 4 in Trial 1 and n = 3 in Trial 2). An SD of 0.0 implies an SD of < 0.05. Values bearing an asterisk (*) between samples of 13 dah ABT larvae (a and b) are significantly different (P < .05). Values of 18 dah ABT larvae (samples c to f) bearing different superscript letters are significantly different (P < .05). DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

[1] Totals include 15:0, 20:0, 22:0 and 24:0.

[2] Totals include 16:1n-9, 18:1n-11, 20:1n-7, 22:1 isomers and 24:1.

[3] Totals include 18:3n-6, 20:2n-6 and 22:4n-6.

[4] Totals include 20:3n-3 and 22:3n-3.

Table 7
Relative abundance (δ) of $^{15}N/^{14}N$ and $^{13}C/^{12}C$ (‰) and C:N ratio of rotifer *Brachionus rotundiformis* enriched with Algamac 3050® (R), nauplii of the copepod *Acartia tonsa* fed on the microalgae *Rhodomonas baltica* (C) and 13 dah Atlantic bluefin tuna (*Thunnus thynnus* L.) larvae from Trial 1 fed on *B. rotundiformis* (ABT R) and *A. tonsa* nauplii (ABT C), and isotopic enrichment (Δ) of ^{15}N and ^{13}C (‰) in ABT larvae in relation to its prey, rotifers and copepods.

R	C	ABT R	ABT C	(e)
$\delta^{15}N$ (‰)	−0.81 ± 0.02	4.39 ± 0.04*	0.65 ± 0.05	7.18 ± 0.01*
$\delta^{13}C$ (‰)	−11.09 ± 0.02	−20.17 ± 0.06*	−10.61 ± 0.17	−17.11 ± 0.01*
C:N	4.64 ± 0.03	3.74 ± 0.02*	3.92 ± 0.01	3.67 ± 0.03*
$\Delta^{15}N$ (‰)			1.46 ± 0.03	2.79 ± 0.02*
$\Delta^{13}C$ (‰)			0.48 ± 0.08	3.06 ± 0.04*

Results are means ± SD (n = 4 in Trial 1 and n = 3 in Trial 2). An SD of 0.0 implies an SD of < 0.05. Values between live prey, or ABT larvae fed on these live prey, bearing an asterisk (*), are significantly different (P < .05).

enrichment (Δ) of ABT larvae in relation to its preys are presented in Table 7. The relative abundance of ^{15}N was significantly higher in *A. tonsa* nauplii than in enriched rotifers, as was the case for 13 dah ABT larvae fed *Acartia* compared to larvae fed enriched rotifer. In contrast, the relative abundance of ^{13}C showed significantly higher values in enriched rotifers than in *Acartia*. The same was observed for ABT larvae, with ABT larvae fed enriched rotifers presenting significantly higher values than larvae fed copepod nauplii. On the other hand, the C:N ratio was significantly higher in enriched rotifers and ABT larvae fed rotifers than in copepod nauplii and ABT larvae fed copepods. The isotopic enrichments $\Delta^{15}N$ and $\Delta^{13}C$ of 13 dah ABT larvae in relation to their preys were also significantly higher in larvae fed copepods than in larvae fed rotifers.

3.8. Gene expression

3.8.1. Myogenic genes

The expression of both *myhc* and *tropo* was significantly higher in ABT larvae fed *A. tonsa* nauplii in Trial 1 (Fig. 1). On the other hand, in Trial 2, the expression of *myhc* in 18 dah ABT larvae was significantly up-regulated in larvae from treatment CY whereas no significant differences were observed among the expression of this gene in larvae from the others treatments (RA, CA and CC). With regard to the expression of *tropo* in 18 dah ABT larvae, no differences were observed among larvae fed the different treatments.

3.8.2. Fatty acid synthesis genes

No significant nutritional regulation was detected at 13 dah

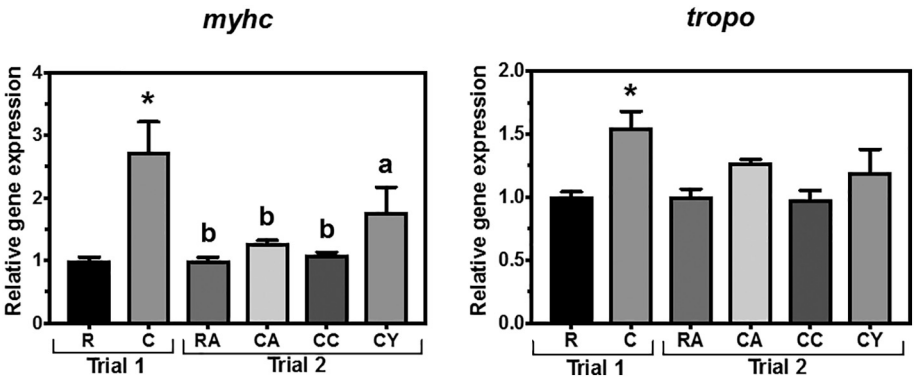

Fig. 1. Nutritional regulation of myosin heavy chain 2 (*myhc*) and tropomyosin (*tropo*) gene transcription in Atlantic bluefin tuna (ABT) larvae 13 days after hatch (Trial 1) fed with rotifer *B. rotundiformis* enriched with Algamac 3050® (R), *A. tonsa* copepod nauplii (C), and ABT larvae 18 days after hatch (Trial 2) fed with rotifer *B. rotundiformis* and then *Artemia* metanauplii enriched with Algamac 3050® (RA), *A. tonsa* copepod nauplii and then *Artemia* enriched with Algamac 3050® (CA), *A. tonsa* copepod nauplii and then followed with *A. tonsa* nauplii and copepodites (CC), and *A. tonsa* nauplii and then gilthead sea bream (*S. aurata*) yolk sac larvae (CY). Values are normalized expression ratios, corresponding to an average of 8 pooled samples in Trial 1 (n = 8) and 6 pooled samples in Trial 2 (n = 6) with standard errors (SEM). Different superscript letters denote differences among the dietary treatments.

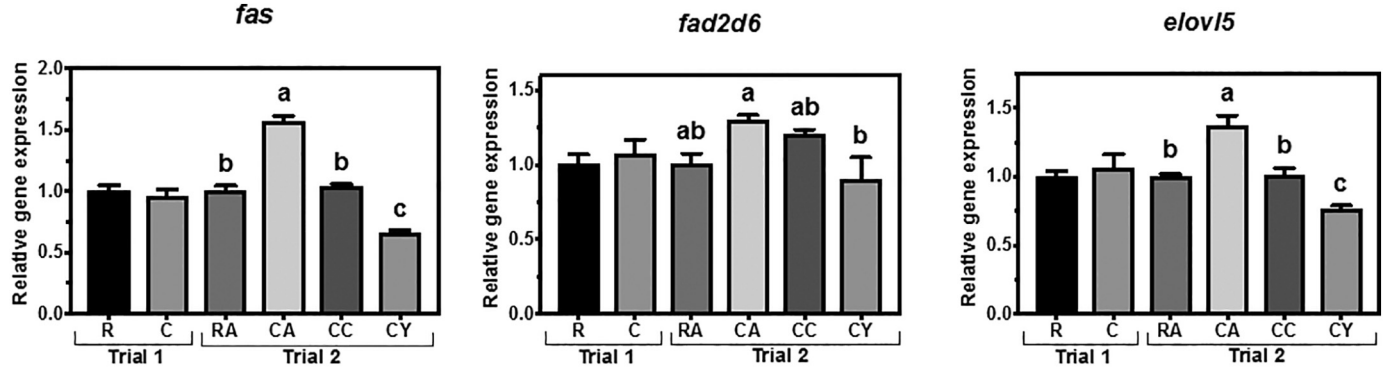

Fig. 2. Nutritional regulation of fatty acid synthase (*fas*), delta-6 fatty acyl desaturase (*fads2d6*) and fatty acyl elongase 5 (*elovl5*) gene transcription in Atlantic bluefin tuna (ABT) larvae 13 days after hatch (Trial 1) fed with rotifer *B. rotundiformis* enriched with Algamac 3050® (R), *A. tonsa* copepod nauplii (C), and ABT larvae 18 days after hatch (Trial 2) fed with rotifer *B. rotundiformis* and then *Artemia* metanauplii enriched with Algamac 3050® (RA), *A. tonsa* copepod nauplii and then *Artemia* enriched with Algamac 3050® (CA), *A. tonsa* copepod nauplii and then followed with *A. tonsa* nauplii and copepodites (CC), and *A. tonsa* nauplii and then gilthead sea bream (*S. aurata*) yolk sac larvae (CY). Values are normalized expression ratios, corresponding to an average of 8 pooled samples in Trial 1 (n = 8) and 6 pooled samples in Trial 2 (n = 6) with standard errors (SEM). Different superscript letters denote differences among the dietary treatments.

between larvae fed on treatments R and C with regard to the relative expression of the 3 fatty acid synthesis genes *fas*, *fad2d6* and *elovl5* (Fig. 2). The expression values for the same 3 genes followed a similar pattern at 18 dah, with ABT larvae fed CA displaying the highest expression levels, albeit not significantly different to expression in larvae fed RA and CC in the case of *fad2d6*. The lowest transcript copy numbers of *fas* and *elovl5* were found in larvae fed CY, whereas the expression of *fad2d6* did not differ between this group and larvae fed RA or CC.

3.8.3. Fatty acid catabolism genes

In relation to fatty acid catabolism, the expression of *cpt1* did not show dietary regulation at 13 dah among larvae fed R or C in Trial 1 (Fig. 3), whereas at 18 dah the highest mRNA copy numbers were observed in larvae fed CY > RA > CA = CC. In contrast, relative expression of *aco* was significantly lower in 13 dah ABT larvae fed treatment C and, in Trial 2, larvae fed CY.

3.8.4. Fatty acid and lipid transport genes

In Trial 1, the relative transcript abundance of *fabp2*, *fabp7* and *lpl* was not significantly different between larvae fed treatments R and C (Fig. 4). In addition, the expression of *fabp4* and *hmgcl* were significantly lower in larvae fed treatment C. In Trial 2, the lowest copy numbers for *fabp2*, *lpl* and *hmgcl* were observed in larvae fed CY, whereas these larvae showed the highest expression levels of *fabp4* and fabp7, albeit not different to those of larvae fed CA in the case of *fabp7* (Fig. 4).

3.8.5. Transcription factors genes regulating lipid metabolism

In Trial 1, the expression levels of *pparα*, *pparγ*, *srebp1* and *srebp2* were significantly lower in larvae fed treatment C (Fig. 5). In contrast, the expression of *rxr* was significantly higher in larvae fed treatment C, whereas no significant differences were observed for expression of *lxr*. In Trial 2, lower expression of *pparα*, *srebp1*, *pparγ* and *lxr* was observed in CY-fed larvae. The relative expression value of *srebp2* was also lowest for larvae fed treatment CY, albeit not different to the level in larvae fed CC. No regulation was observed in the expression levels of *rxr* at 18 dah (Fig. 5).

3.8.6. Antioxidant defence enzyme genes

In Trial 1, the expressions of *sod*, *gpx1* and *gpx4* were significantly lower in larvae fed *Acartia* nauplii (C), whereas the expression of *cat* was not regulated between larvae fed treatments R or C (Fig. 6). In Trial 2, *sod* expression was significantly higher in ABT larvae fed treatment CY, whereas abundance of *cat* and *gpx4* was lower in the same larvae. The highest *gpx1* mRNA copy number was found in larvae fed RA, albeit not different to that of larvae fed CY (Fig. 6).

3.8.7. Digestive enzyme genes

In Trial 1, the expression values of *tryp*, *alp*, and *bal1* showed no regulation between 13 dah ABT larvae fed treatments R or C (Fig. 7). In contrast, the expression levels of *anpep*, *amy*, *pl*, *pla2* and *bal2* were significantly lower in ABT larvae fed treatment C. In contrast, in Trial 2, larvae fed CY displayed the lowest expression levels for all the digestive genes, excepting for *alp*, where no differences were found among larvae

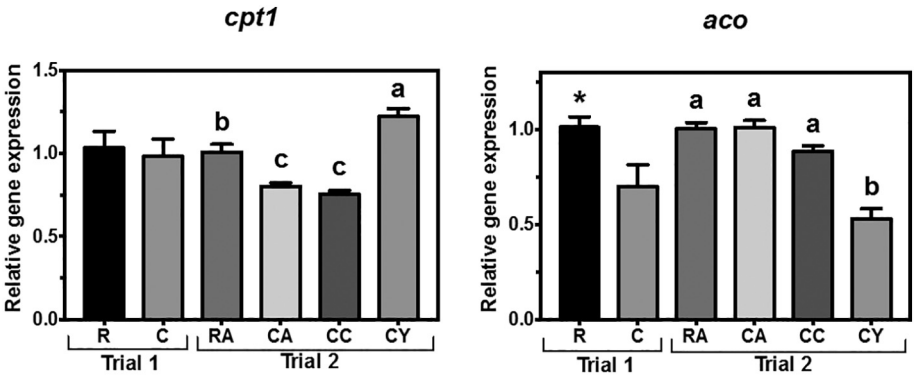

Fig. 3. Nutritional regulation of carnitine palmitoyl transferase I (*cptI*) and acyl coA oxidase (*aco*) gene transcription in Atlantic bluefin tuna ((ABT) larvae 13 days after hatch (Trial 1) fed with rotifer *B. rotundiformis* enriched with Algamac 3050® (R), *A. tonsa* copepod nauplii (C), and ABT larvae 18 days after hatch (Trial 2) fed with rotifer *B. rotundiformis* and then *Artemia* metanauplii enriched with Algamac 3050® (RA), *A. tonsa* copepod nauplii and then *Artemia* enriched with Algamac 3050® (CA), *A. tonsa* copepod nauplii and then followed with *A. tonsa* nauplii and copepodites (CC), and *A. tonsa* nauplii and then gilthead sea bream (*S. aurata*) yolk sac larvae (CY). Values are normalized expression ratios, corresponding to an average of 8 pooled samples in Trial 1 (n = 8) and 6 pooled samples in Trial 2 (n = 6) with standard errors (SEM). Different superscript letters denote differences among the dietary treatments.

fed the different dietary treatments. ABT larvae fed treatment CC displayed the highest expression levels of *tryp, pla2 bal1* and *bal2* (Fig. 7).

4. Discussion

Previous studies had shown that copepods supported better growth performance than other live prey for larvae of ABT at first feeding (Yufera et al., 2014; Betancor et al., 2017a,b). The present study was consistent with these data, as larvae fed this live prey displayed larger biometric data, advanced stage of development (highest flexion index), and highest expression of the myogenic genes, *myhc* and *tropo* than ABT larvae fed on enriched rotifer at 13 dah. Additionally, in the present study we evaluated the effect that different live prey can have on later stages (18 dah) of ABT larvae performance in combination with the earlier use of either rotifers or copepods. A higher C:N ratio in the rotifer coincides with a significantly higher total lipid content in this prey than in the copepod, as was the case for rotifer-fed 13 dah ABT larvae, whose average total lipid content was higher than ABT larvae fed copepods, albeit not statistically significant. The δ^{15}N has been used to examine the trophic position of organisms in food webs (Owens, 1987; Hobson and Welch, 1992; Michener and Kaufman, 2007), with ^{15}N values of consumers generally increasing with trophic level, with an

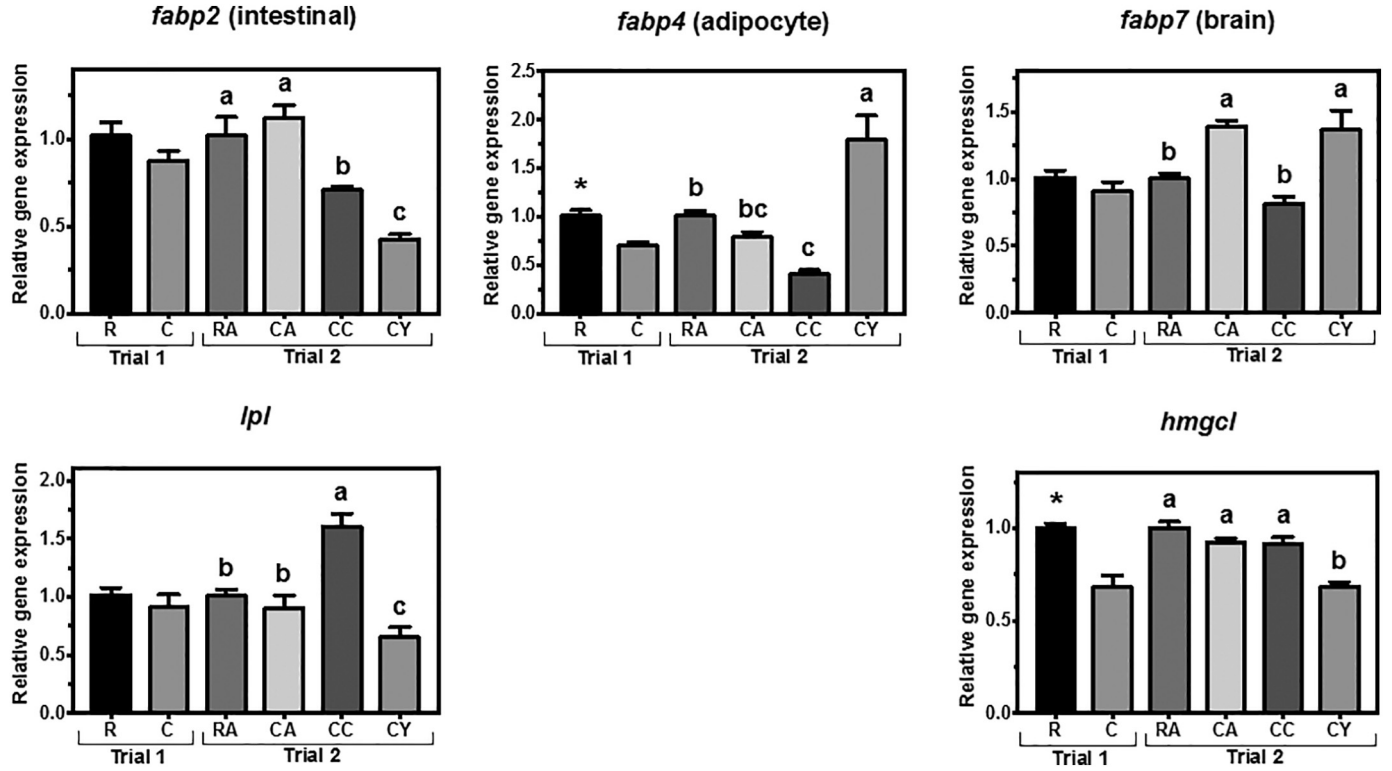

Fig. 4. Nutritional regulation of fatty acid binding protein 2, 4 and 6 (*fabp2, fabp4* and *fabp7* respectively), lipoprotein lipase (*lpl*) and 3-hydroxy-3-methylglutaryl-CoA lyase (*hmgcl*) gene transcription in Atlantic bluefin tuna (ABT) larvae 13 days after hatch (Trial 1) fed with rotifer *B. rotundiformis* enriched with Algamac 3050® (R), *A. tonsa* copepod nauplii (C), and ABT larvae 18 days after hatch (Trial 2) fed with rotifer *B. rotundiformis* and then *Artemia* metanauplii enriched with Algamac 3050® (RA), *A. tonsa* copepod nauplii and then *Artemia* enriched with Algamac 3050® (CA), *A. tonsa* copepod nauplii and then followed with *A. tonsa* nauplii and copepodites (CC), and *A. tonsa* nauplii and then gilthead sea bream (*S. aurata*) yolk sac larvae (CY). Values are normalized expression ratios, corresponding to an average of 8 pooled samples in Trial 1 (n = 8) and 6 pooled samples in Trial 2 (n = 6) with standard errors (SEM). Different superscript letters denote differences among the dietary treatments.

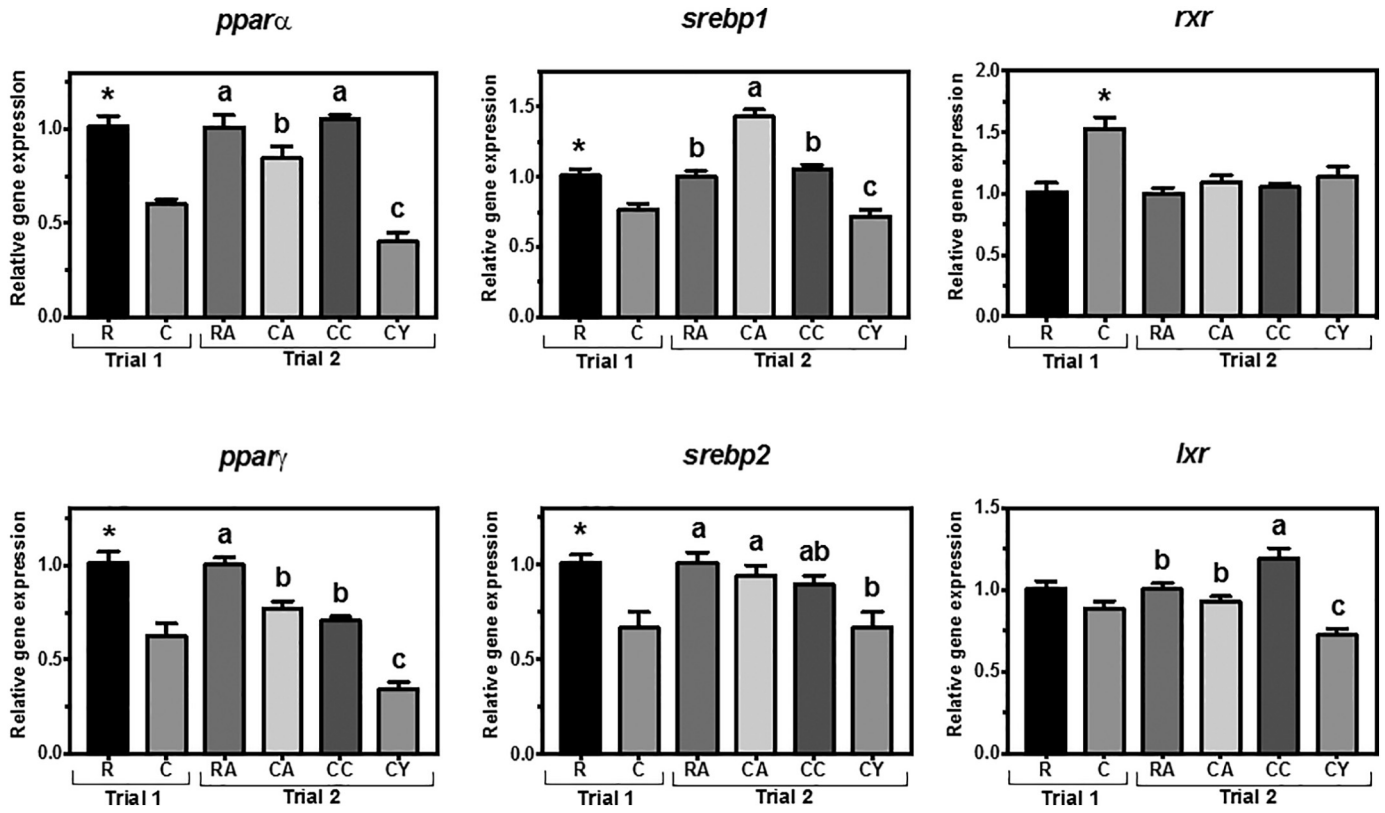

Fig. 5. Nutritional regulation of peroxisome proliferator-activated receptor alpha (*pparα*), gamma (*pparγ*), sterol regulatory element-binding protein 1 and 2 (*srebp1*and *srebp2* respectively), retinoid X receptor (*rxr*) and liver X receptor (*lxr*) gene transcription in Atlantic bluefin tuna (ABT) larvae 13 days after hatch (Trial 1) fed with rotifer *B. rotundiformis* enriched with Algamac 3050® (R), *A. tonsa* copepod nauplii (C), and ABT larvae 18 days after hatch (Trial 2) fed with rotifer *B. rotundiformis* and then *Artemia* metanauplii enriched with Algamac 3050® (RA), *A. tonsa* copepod nauplii and then *Artemia* enriched with Algamac 3050® (CA), *A. tonsa* copepod nauplii and then followed with *A. tonsa* nauplii and copepodites (CC), and *A. tonsa* nauplii and then gilthead sea bream (*S. aurata*) yolk sac larvae (CY). Values are normalized expression ratios, corresponding to an average of 8 pooled samples in Trial 1 (n = 8) and 6 pooled samples in Trial 2 (n = 6) with standard errors (SEM). Different superscript letters denote differences among the dietary treatments.

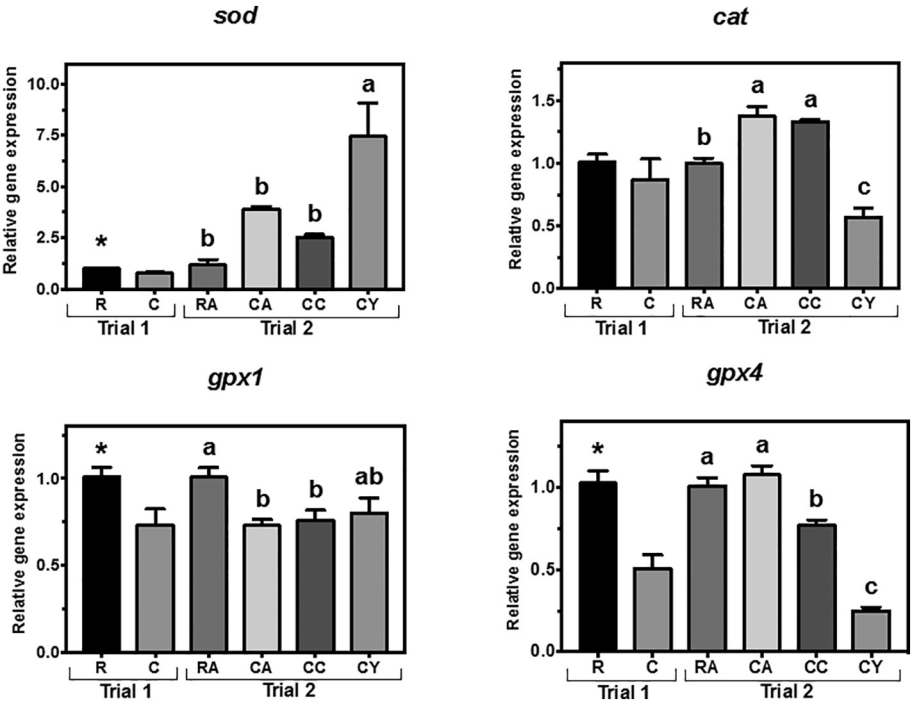

Fig. 6. Nutritional regulation of superoxide dismutase (*sod*), catalase (*cat*) and glutathione peroxidase 1 and 4 (*gpx1* and *gpx4* respectively) gene transcription in Atlantic bluefin tuna (ABT) larvae 13 days after hatch (Trial 1) fed with rotifer *B. rotundiformis* enriched with Algamac 3050® (R), *A. tonsa* copepod nauplii (C), and ABT larvae 18 days after hatch (Trial 2) fed with rotifer *B. rotundiformis* and then *Artemia* metanauplii enriched with Algamac 3050® (RA), *A. tonsa* copepod nauplii and then *Artemia* enriched with Algamac 3050® (CA), *A. tonsa* copepod nauplii and then followed with *A. tonsa* nauplii and copepodites (CC), and *A. tonsa* nauplii and then gilthead sea bream (*S. aurata*) yolk sac larvae (CY). Values are normalized expression ratios, corresponding to an average of 8 pooled samples in Trial 1 (n = 8) and 6 pooled samples in Trial 2 (n = 6) with standard errors (SEM). Different superscript letters denote differences among the dietary treatments.

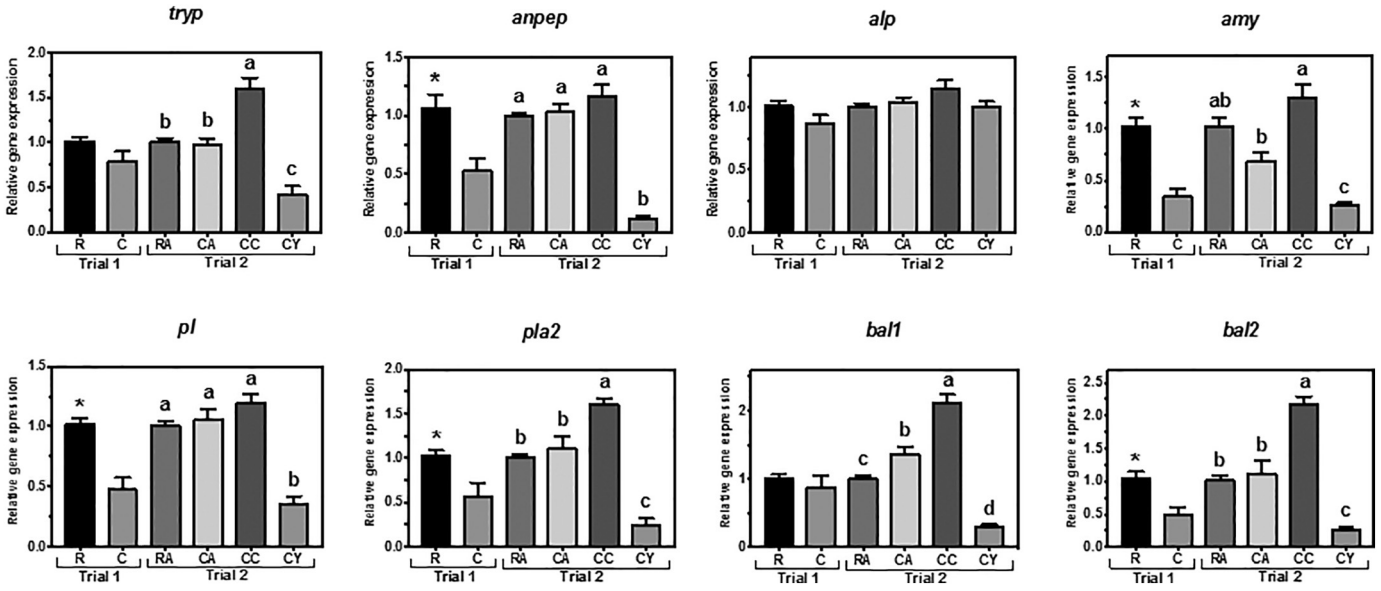

Fig. 7. Nutritional regulation of trypsin (*tryp*), amino peptidase (*anpep*), amylase (*amy*), pancreatic lipase (*pl*), phospholipase A_2 (*pla2*) and bile salt activated lipase 1 and 2 (*bal1* and *bal2* respectively) gene transcription in Atlantic bluefin tuna (ABT) larvae 13 days after hatch (Trial 1) fed with rotifer *B. rotundiformis* enriched with Algamac 3050® (R), *A. tonsa* copepod nauplii (C), and ABT larvae 18 days after hatch (Trial 2) fed with rotifer *B. rotundiformis* and then *Artemia* metanauplii enriched with Algamac 3050® (RA), *A. tonsa* copepod nauplii and then *Artemia* enriched with Algamac 3050® (CA), *A. tonsa* copepod nauplii and then followed with *A. tonsa* nauplii and copepodites (CC), and *A. tonsa* nauplii and then gilthead sea bream (*S. aurata*) yolk sac larvae (CY). Values are normalized expression ratios, corresponding to an average of 8 pooled samples in Trial 1 (n = 8) and 6 pooled samples in Trial 2 (n = 6) with standard errors (SEM). Different superscript letters denote differences among the dietary treatments.

average 3.2‰ of enrichment per trophic level (Michener and Kaufman, 2007). In the present study, ABT larvae fed *A. tonsa* nauplii showed higher values of enrichment of N (Δ^{15}N) and C (Δ^{13}C) than ABT larvae fed on enriched *B. rotundiformis* (2.79 *vs.* 1.46 for Δ^{15}N, and 3.06 *vs.* 0.48 for Δ^{13}C, respectively). This is also consistent with *Acartia* nauplii having a significantly higher content of total protein (63.2% *vs.* 51.3% on a dry matter basis) and total amino acids (571.5 *vs.* 334.3 mg g^{-1} dry mass) than rotifers. Gilthead sea bream yolk sac larvae, as a live prey showed the highest content of essential amino acids with indication of a slight deficiency only for valine. Thus, the stable isotope data support growth performance data, where ABT larvae fed on copepods grew and developed faster than ABT larvae fed on enriched rotifers as they were prey of a supposedly "higher trophic position", and had better nutritional value, in consequence fixing more N and C.

Fast growth in ABT larvae during early life stages, under standard rearing conditions, occurs after notochord flexion, when piscivory is attained (Uriarte et al., 2016). The results of the present study showed that ABT larvae fed *Acartia* nauplii attained 54.5% of flexioned larvae *versus* only 35.8% for ABT larvae fed enriched rotifers at 13 dah. This implies an advantage to reach the faster growth phase after flexion with larvae that had been fed copepods attaining higher growth rates than those fed rotifers at 18 dah. Indeed, highest growth was obtained in ABT larvae fed copepods first (to 13 dah) and then on gilthead sea bream yolk sac larvae (to 18 dah), reflecting the contribution of piscivory in the diet. Once ABT larvae attained piscivory, a very high growth performance was obtained, in agreement with previous reports with Pacific Bluefin tuna (PBT; *Thunnus orientalis*) larvae (Tanaka et al., 2007, 2010, 2014, 2015). Moreover, results on PBT larvae suggested that fast-growing larvae at the onset of piscivory could survive in the mass culture tank and were characterized by growth-selective mortality based on direct evidence by comparing feeding (nitrogen stable isotope ratios) and growth between live and dead fish (Tanaka et al., 2017).

In the present trial, survival was not different among treatments at 13 dah, since the variability of average survival rate among replicates was relatively large. Such a difference in survival is currently the norm in the tuna hatchery phase at the present time and limited the number of fish available for the second phase of the trial, 13–18 dph, which was cosequently carried out in single replicate tanks to provide fish density to promote adequate feeding response. Great differences in survival/growth have been apparent among different cohorts of fish produced since the first eggs were hatched in 2009. These differences appear related to broodstock, as presently eggs are collected from floating cages and therefore there is no accurate control of each broodstock contribution. However, the mortality of ABT larvae at first feeding should not be quantitatively related to the nutritional quality of the live prey. Survival of tuna larvae in artificial rearing has been reported to be relatively low compared to other fish species (Miyashita, 2002). Multiple factors are thought to contribute to mortality of larval tuna in rearing systems during the early larval stages, such as suboptimal physical condition, including increased bacterial loads, surface adhesion and, of course, malnutrition as an added negative effect (Margulies et al., 2016; Honryo et al., 2017). At these early stages, the vitality and strength of the larvae also depends to a great extent on broodstock nutrition and the quality of the eggs and yolk sac larvae produced, and this may vary with and within every spawned batch. Some other neglected zootechnical factors dealing with rearing conditions in individual replicates/tanks may affect swim bladder inflation and larval survival. Besides, mortality and poor development during larval stages has also been attributed to antioxidant status (Mourente et al., 1999; Fernandez-Diaz et al., 2006; Mazurais et al., 2015; Penglase et al., 2015), as discussed below. In any case, the provision of a high-quality nutritional enhancement of planktonic prey during ABT larvae first feeding, a period of high metabolic demand required to support exponential growth, is of paramount importance.

Dietary fatty acid composition is known to have a modulatory effect on enzyme activities controlling fatty acid biosynthesis and bioconversion pathways in cultured teleost species (Zheng et al., 2004; Thanuthong et al., 2011; Ayisi et al., 2018). In fish, fatty acids can arise from two sources; either synthesized *de novo* from non-lipid carbon sources, or directly from dietary lipid. Fatty acid synthase (Fas) plays a key role in the process of *de novo* lipogenesis, and the rate of fatty acid *de novo* synthesis has been shown to be negatively correlated to the

level of dietary lipids (Henderson, 1996). In trial 2 in the present study, there was a strong negative correlation between dietary total lipid content and *fas* expression (r = −0.63; P = .05), indicating and inhibition of *fas* expression by increased dietary lipid levels, as stated above. In contrast, in Trial 1, *fas* expression showed no differences in larvae fed on either rotifers or copepods, possibly due to sufficient lipid content in these preys. On the other hand, *fas* is a direct target gene of the transcription factor *srebp1*, and both genes followed a similar pattern of expression (r = 1.0; P = .08). These data were in contrast to the results of our previous studies with ABT larvae (Betancor et al., 2017a, 2017b).

As a general consideration, increased expression of *fads2d6* has been observed previously in fish fed low levels of dietary n-3 LC-PUFA, whereas high dietary levels of these fatty acids has been associated with reduced relative transcript abundance (Morais et al., 2012; Betancor et al., 2015). In the present study, in Trial 1, no dietary regulation was detected at 13 dah among ABT larvae fed treatments R or C with regard to the relative expression of the 3 fatty acid synthesis genes *fas*, *fas2d6* and *elovl5*. These results could be interpreted in the following manner: i) both prey (enriched rotifers and copepod nauplii) supplied sufficient lipid to maintain *fas* expression at a baseline level and ii) both prey supplied sufficient n-3 and n-6 PUFA to keep *fad2d6* and *elovl5* expression at a baseline level. This may also mean that prey lipid and essential fatty acid contents in Trial 1 satisfied ABT larvae requirements for these nutrients at first feeding and did not significantly affect fatty acid or LC-PUFA synthesis gene expression. In contrast, in Trial 2, the expression of *fad2d6* in 18 dah ABT larvae was higher in larvae fed enriched *Artemia* for 5 days after feeding on copepod nauplii diet (treatment CA), and those fed treatments RA and CC were also higher than larvae fed CY. This seems logical taking into account that the *Artemia* diet presented the lowest level of total n-3 PUFA and DHA. In contrast, ABT larvae fed treatment CY showed lower expression of *fads2d6*, possibly due to higher levels of DHA and total n-3 PUFA in both copepod nauplii and sea bream yolk sac larvae than other live prey. A similar pattern of expression to dietary treatments was shown by *elolv5* in 18 dah ABT larvae, which further supports up-regulation of the LC-PUFA pathway in response to the low dietary LC-PUFA in treatment *CA*. This suggests that n-3 LC-PUFA requirement of 18 dah ABT larvae could be above the level supplied by treatment CA (mainly *Artemia*) and below or similar to that of CY. In agreement, a previous study showed that an increased DHA/EPA ratio in ABT larvae in parallel with increased expression of *fad2d6* and *elovl5* during development of unfed ABT larvae was associated with elongation and desaturation of EPA in order to maintain adequate DHA levels (Morais et al., 2011).

High dietary levels of n-3 LC-PUFA, particularly DHA, can act as ligands for transcription factors such as *ppara* and *srebp1*, down-regulating the biosynthesis of LC-PUFA (Worgall et al., 1998; Hihi et al., 2002; Cunha et al., 2013; Peng et al., 2014), and regulating the expression of their target genes such as *fas*, *cptI*, *aco* or *lpl*. In the present study, *srebp1* was strongly positively correlated with the expression of *fas* (r = 1.0; P = .08) and *elovl5* (r = 1.0; P = .08) in Trial 2. However, no correlation was observed in Trial 2 between dietary DHA levels and the expression values of *ppara* and *srebp1*. Previous studies in teleosts have described an inhibition in hepatic *fas* expression when fish were fed in a restricted manner (Tian et al., 2013; He et al., 2015; Gong et al., 2017) or with increased dietary lipid (Leng et al., 2012), including our previous results with ABT larvae at first feeding (Betancor et al., 2017b). Although the transcription factor genes *ppara* and *srebp1* did not show nutritional regulation in Trials 1 and 2, *fas*, a direct target of *srebp1*, was strongly regulated by the different dietary treatments in Trial 2 and followed a similar pattern of expression as *srebp1* in response to diet.

Pparγ, a transcription factor involved in adipocyte function and differentiation and lipid storage by adipocytes (Nedergaard et al., 2005; Ji et al., 2011; Agawa et al., 2012; Ayisi et al., 2018), showed lower expression in ABT larvae fed copepods in Trial 1 and 2, with its expression negatively related to lipid content. Thus, taking into account that copepods are among the natural live prey of ABT larvae in the wild (Uotani et al., 1990; Catalán et al., 2011; Tilley et al., 2016; Kodama et al., 2017), it is feasible to suggest that the high lipid content of enriched rotifers (treatment R in Trial 1 and treatment RA in Trial 2) triggered a response in ABT larvae that modulated lipogenetic/lipolytic mechanisms in order to adapt to an energy-dense ration. In this sense, lower *pparγ* expression was also observed in copepod-fed ABT larvae in previous trials (Betancor et al., 2017a, 2017b). In mammals, targets directly regulated by *pparγ* include genes that favour uptake of circulating fatty acids by adipocytes (Schoonjans et al., 1996; Frohnert et al., 1999; Chui et al., 2005) and others that promote recycling rather than export of intracellular fatty acids (Guan et al., 2002; Hibuse et al., 2005). These paradoxical effects on adipocyte biology means that, apart from enhancing fatty acid deposition similar to *ppara*, *pparγ* can lead to increased fatty acid oxidation (Lehrke and Lazar, 2005). The β-oxidation of fatty acids takes place in both mitochondria and peroxisomes, but mitochondrial β-oxidation is quantitatively more important and can utilize a wider range of fatty acids as substrate (Henderson, 1996). In this respect, *pparγ* expression was down-regulated by dietary treatments with higher n-3 LC-PUFA (CA, CC and CY) and the same trend was followed by the expression of *aco* in Trial 2. Nevertheless, the *cpt1* expression pattern for larvae fed this treatment was the opposite, being higher in ABT larvae fed yolk sac larvae. CPT-1 is considered to be the main regulatory enzyme in long-chain fatty acid β-oxidation and the higher expression observed in larvae fed yolk sac larvae might be explained by the superior content of LC-PUFA in this live prey.

SREBP1 preferentially regulates fatty acid and LC-PUFA synthesis, whereas SREBP2 regulates the expression of genes involved in cholesterol synthesis (Jeon and Osborne, 2012; Carmona-Antoñanzas et al., 2014) and is up-regulated in response to reduced cholesterol (Minghetti et al., 2011; Carmona-Antoñanzas et al., 2014). Consistent with this, in the present study, there was an inverse correlation between cholesterol levels in enriched rotifers and copepods and *srebp2* expression in ABT larvae in Trial 1. Moreover, in Trial 2, the expression of *srebp2* was also negatively correlated (r = −0.95; P = .17) to dietary cholesterol levels.

LXR is important in controlling intermediary metabolism mediating cross-regulation between sterol and fatty acid metabolism (Ayisi et al., 2018), and its ligands can be antagonized by fatty acids (Cruz-Garcia et al., 2012). Thus, high levels of unsaturated fatty acids activate *lxr,* whereas activated *lxr* induces cholesterol catabolism and *de novo* fatty acid biosynthesis in liver, which has led to the suggestion that *lxrs* are sensors of the balance between cholesterol and fatty acid metabolism (Ayisi et al., 2018). In Trial 2, a weak negative correlation (r = −0.32; P = .67) was observed between dietary cholesterol levels and *lxr* expression. Although there was no significant correlation between dietary cholesterol levels and *lxr* expression in Trial 2, *lxr* expression was positively correlated with the expression of the bile acid activated lipases *bal1* and *bal2*, (r = 0.80; P = .33). The lack of a clear relation between dietary cholesterol and *lxr* expression may be due to the fact that LXR is activated by several sterols, including intermediates in the synthesis of cholesterol (Carmona-Antoñanzas et al., 2014) and, although the level of cholesterol differed among treatments, the levels of other sterols that may activate *lxr* might be similar. In addition to the role of *lxr* in regulating cholesterol catabolism, storage, absorption and transport through the transcriptional regulation of key target genes involved in these processes, it may also act in fatty acid metabolism by increasing the expression of the transcription factor *srebp1* or genes such as *fas* or *lpl*. In this sense, the mRNA levels of *lxr* and *lpl* showed a strong positive correlation (r = 1.0; P = .08) and the same pattern of expression in 18 dah ABT larvae. Lpl is a lipase highly expressed in muscle and liver of ABT (Betancor et al., 2017a) that hydrolyzes TAG in plasma lipoproteins and supplies free fatty acids for deposition in adipose tissue or for oxidation in other tissues (Nilsson-Ehle et al., 1980; Kersten, 2014; Ayisi et al., 2018). In agreement, high levels of *lpl* expression and activity have been associated with increased lipid utilization in

darkbarbel catfish (*Pelteobagrus vachelli*) larvae fed high-lipid diets (Zheng et al., 2010).

Fatty acid binding proteins (Fabp) in general are noted for the intracellular transport of fatty acids and play an intermediary role in orchestrating gene transcription involved in lipid homeostasis (Tocher, 2003; Ayisi et al., 2018). In agreement with our previous studies (Betancor et al., 2017a, 2017b), *fabp4*, a carrier protein involved in fatty acid uptake, transport and metabolism in adipocytes (Glatz and van der Vusse, 1996), was down-regulated in copepod-fed ABT larvae in Trial 1, possibly reflecting increased uptake and accumulation of lipid into larval tissues. On the other hand, *fabp2* (intestinal) and *fabp7* (brain) showed no differences in their expression between 13 dah ABT larvae fed treatments R or C. In agreement, a recent study in Senegalese sole (*Solea senegalensis*) larvae showed no regulation of *fabp2* expression when larvae were fed enriched *Artemia*, whereas up-regulation of *fabp1* and *fabp3* was observed in larvae fed high levels of n-3 LC-PUFA (Bonacic et al., 2016), which may indicate differential regulation of *fabp* at different developmental stages (André et al., 2000). In Trial 2 in the present study, different patterns of regulation were presented by different *fabp* genes in response to dietary treatments. However, the positive correlation ($r = 0.80$; $P = .33$) between *fas* expression and *fabp2* (intestinal) expression in ABT larvae fed the different dietary treatments in Trial 2 was noteworthy.

Rotifers and *Artemia* have low levels of several nutrients, including antioxidants, compared to copepods (Hamre et al., 2013). Nutrient intake plays a role in regulating the redox system in fish larvae, suggesting that nutrient-induced changes in the redox system may contribute to differences in larval fish growth and development (Mourente et al., 2007; Izquierdo and Betancor, 2015; Penglase et al., 2015). In Trial 1, C-fed larvae showed lower expression of *sod*, *gpx1* and *gpx4*, possibly in response to lower contents of antioxidant nutrients such as Se or vitamin E. It was more complicated in Trial 2 as the pattern of expression was variable among the different dietary treatments. Differences could not only be attributed to the feed, but also to the stage of development of the larvae as it is known that lipid peroxidation levels in larvae show diet and age/growth dependence in their responses (Fernandez-Diaz et al., 2006). Therefore, the differences in growth among the larvae could influence lipid oxidation levels. It is perhaps noteworthy that the Se content of live prey, either enriched rotifers or *Acartia* nauplii, showed a significant negative correlation with final total length ($r = -0.9$; $P = .0417$), total weight ($r = -0.9$; $P = .008$) and flexion index ($r = -0.8$; $P = .053$). Although Se is an essential micronutrient, it has the narrowest window of any element between requirement and toxicity (Polatajko et al., 2006), with reduced growth being one of the first symptons of toxicity (Jaramillo et al., 2009). Thus, it would need to be established whether the Se levels used in enriched rotifers in the present study were within safe limits for ABT larvae.

Molecular methodologies could contribute to the understanding of the real digestive capacities of developing larvae under different dietary protocols. In the present study, expression of trypsin (*tryp*), pancreatic alkaline protease (*alp*) and bile acid activated lipase 1 (*bal1*) was not regulated by dietary treatments in Trial 1. In contrast, in Trial 2, all digestive anzyme genes studied were significantly up-regulated by treatment CC and down-regulated by treatment CY, which were the largest larvae, which could indeed indicate that the expression levels are related to the developmental stage. It is commonly accepted that the major digestive lipase in teleosts, including larvae, appears to be bile salt-dependent lipases (*bal*) (Rønnestad and Morais, 2008). It was also reported that *Bal* were the main enzymes involved in lipid digestion in the larval stage of PBT (Murashita et al., 2014). In the present study, the expression patterns of both isoforms (*bal1* and *bal2*) were nutritionally regulated, showing a similar pattern.

In conclusion, and in agreement with our previous trials, the present study showed that copepods (*Acartia*) were a superior live prey for first feeding ABT larvae compared to enriched rotifers, as indicated by higher growth performance. This rapid growth would enable rapid attainment of the piscivory feeding stage, which would also enable earlier weaning to inert formulated diet. This may reflect the higher protein and essential amino acids, polar lipid and n-3 LC PUFA and other nutrient contents of copepods as first prey. When the start of piscivory is delayed (larvae fed Artemia or copepods) larvae previously fed copepods do not show any advantage in growth compared to larvae fed with rotifers. The highest DHA contents were found in ABT larvae fed CC and CY, whereas the lowest contents were found in RA-fed larvae. Although, RA-fed larvae showed the highest level of the intermediate product n-3 DPA, this was not supported by gene expression data. Different expression patterns of digestive enzymes between ABT larvae fed copepods and enriched rotifers could be due to different lipid class/fatty acid compositions of the live prey or to differences in the size/development of the larvae.

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